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Preparation and characterization of defined conjugates of glucose oxidase and peroxidase.

Bouchra. Haraké
University of Windsor

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PREPARATION AND CHARACTERIZATION OF DEFINED CONJUGATES OF GLUCOSE OXIDASE AND PEROXIDASE

by

BOUCHRA HARAKÉ

A THESIS

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ABSTRACT

PREPARATION AND CHARACTERIZATION OF DEFINED CONJUGATES OF GLUCOSE OXIDASE AND PEROXIDASE

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BOUCHRA HARAKÉ

The conjugation of glucose oxidase (GO) from *Asperigillus niger* and horseradish peroxidase (HRP) is described.

Conjugation of the two glycoenzymes was carried out by Schiff base formation between the acylhydrazide groups of adipic dihydrazide (ADH) and aldehydic functions derived from mild periodate-oxidized preparations of peroxidase and glucose oxidase. The ADH was incorporated into oxidized GO to form a hydrazone with a distal free hydrazide, which was then conjugated with oxidized peroxidase to form glucose-oxidase-dihydrazone-peroxidase (GO-DH-HRP) conjugate. The amount of hydrazide groups incorporated into oxidized enzyme was determined by a 2,4,6-trinitrobenzenesulfonic acid (TNBS) titration.

Quantitation of enzyme activities before and after conjugation was carried out by measurement of the catalyzed rate of oxidative coupling of 2-hydroxy-3,5-dichlorobenzenesulfonate (HDCBS) and 4-aminoantipyrene (4-AAP). Peroxidase activities were measured in the presence of hydrogen peroxide as a substrate, while glucose oxidase activities were determined with glucose as a substrate and in the presence of free peroxidase. The conjugate activity was measured using glucose as a substrate however, no free peroxidase was added to the system.

Kinetic evaluation of the enzymes before and after conjugation was carried out on the different preparations and compared to their native forms. The apparent K_m for conjugated peroxidase was less than that of the native enzyme,

and constant for conjugated glucose oxidase. The relative specific activities of the conjugated enzyme compared to the respective native forms were maintained at an average of 72 and 92% for peroxidase and glucose oxidase, respectively. The conjugated enzymes were found to be as stable as the respective native enzymes.

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I am indebted to my close friend Dr. J.C.K. Yee for his support and encouragement. Finally, I would like to thank my friends and colleagues in the Department of Chemistry and Biochemistry for their moral support and companionship.

DEDICATION

to

my parents

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4-AAP	4-aminoantipyrene
ADH	adipic dihydrazide
GO	Glucose Oxidase
HDCBS	2-hydroxy-3,5-dichlorobenzenesulfonate
HRP	Horseradish Peroxidase
h	hour
PAGE	Polyacrylamide gel electrophoresis
RT	room temperature
RZ	Reinheitszahl
SDS	Sodium Dodecyl Sulfate
SD	standard deviation
TNBS	2,4,6-trinitrobenzenesulfonic acid
$\bar{x}$	mean

CHAPTER I

INTRODUCTION

For various biochemical studies it will be useful to have simple methods for preparing conjugates of one protein with another protein or peptide. The first application, an enzyme-antibody conjugate for use in an immunoassay was reported in 1972 (1). Since then techniques for protein-protein conjugation have been developed. The most widely used method for this purpose involves the coupling of two components by reaction with glutaraldehyde (2,3) to give a mixture of conjugates of like and unlike components. To avoid formation of conjugates of like components, one uses two components with different reactive groups for the coupling reaction. Three reported methods utilizing this principle involve the reaction of a sulfhydryl group of one protein with a haloacetyl group (4,5) a maleimido group (6), or a 4-dithiopyridyl group (7,8) of another protein or peptide. King et al. (9) prepared protein-protein conjugates by modifying amino groups on one to carry arylaldehyde and on the other to carry an acylhydrazide in order to form a hydrazone linkage. Others utilized the carboxyl groups of glutamic and aspartic acid residues (10). One feature common to all of these approaches is the modification of amino acid side chains, which results in a lack of specificity of the reactions in that the conjugation cannot be directed to an amino acid at particular site(s) on the protein(s). In addition, if the conjugates are antibody-enzyme ones, a decrease in the affinity of the antibody for the antigen and/or a decrease in the activity of the enzyme may occur for the above-mentioned modification. Such problems may be avoided by making use of other functionalities available on the enzyme or antibody.

Researchers have shown that the carbohydrate content of some glycoproteins is not essential to the catalytic properties of the enzyme (11-14). Conjugation through these residues is a viable alternative to the modification of amino acid side chains. A few reports have appeared in the literature utilizing the oligosaccharide moieties of immunoglobulins to attach to the amino groups of an enzyme or vice versa (11,12,15-17). Others utilized hydrazone formation to prepare conjugates of proteins with various ligands (10,15,16,18-21). In those studies, glycoproteins were either chemically or enzymatically oxidized to generate aldehyde groups and then coupled to low molecular weight ligands containing various types of amino groups. This general approach is demonstrated in Figure 1, where conjugation of two glycoproteins is carried out by Schiff base formation (Figure 1A) between an amino group of a reporter molecule and the periodate treated enzyme or antibody. The Schiff base needs to be stabilized by sodium cyanoborohydride ($NaBH_3CN$) or borohydride ($NaBH_4$). Reduction of the imine with the former is generally achieved at neutral pH, while sodium borohydride reaction proceeds at more alkaline pH (22,23).

The second type of reporter molecules are those containing hydrazino groups (Figure 1B). These offer several advantages over the use of amino compounds. Firstly, the pKa of a hydrazide is approximately 2.6 (24), whereas that of an amine is 8-9. Therefore, the use of hydrazides allows for the labelling reaction to be carried out under conditions (neutral or slightly acidic pH) that prevent the conjugation of the ϵ-amino groups on one enzyme to the aldehyde groups on the other. Secondly, the reaction product of a hydrazide and an aldehyde, a hydrazone, is stable and does not require reduction with cyanoborohydride thereby circumventing one of the reactions associated with labelling with simple amino compounds.

FIGURE 1

CONJUGATION OF GLYCOPROTEINS THROUGH THEIR CARBOHYDRATE MOIETIES

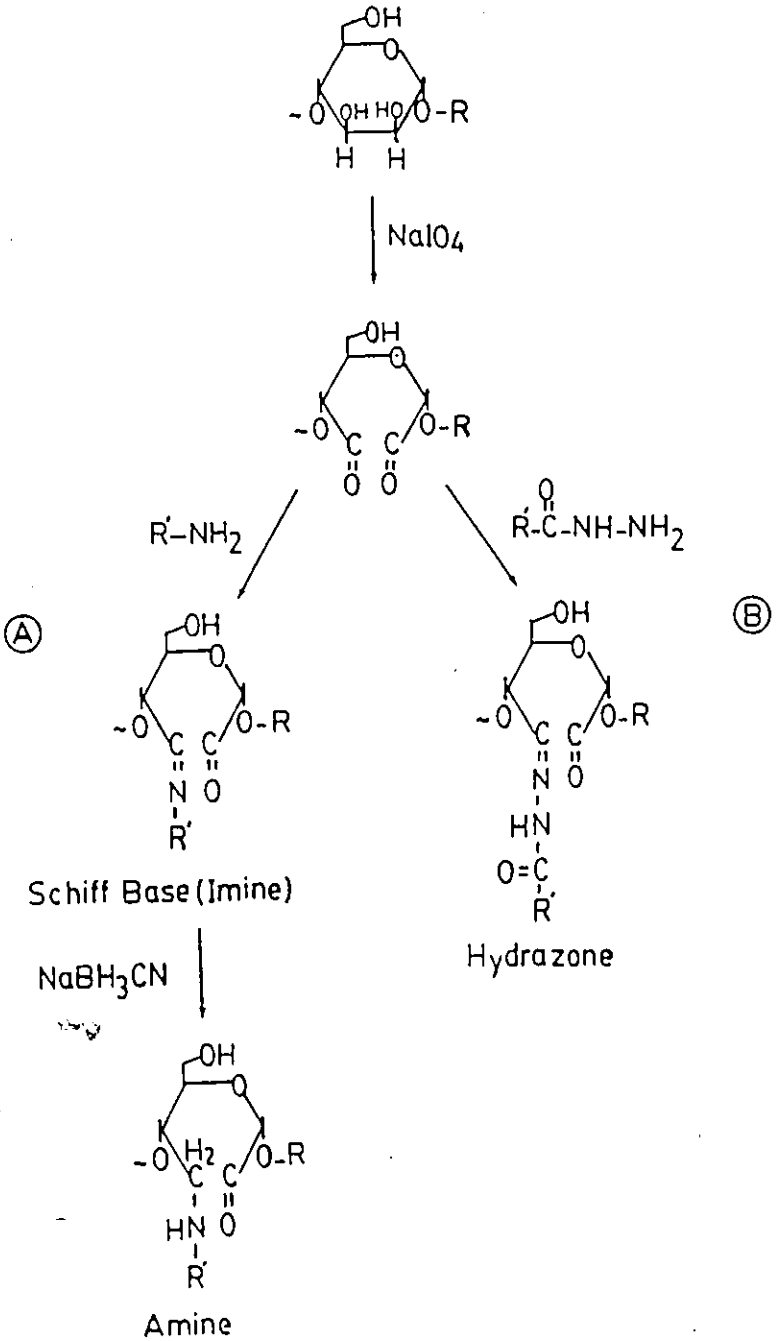
Legend

The general chemistry involved in the site-specific modification or conjugation of the oligosaccharide moieties of glycoproteins is illustrated. Mild periodate oxidation results in the generation of aldehyde groups which can then be condensed specifically with an amine or hydrazide.

R = Antibody or enzyme molecule

R^1 = Reporter molecule such as: drug, biotin, toxin, radiolabel or fluorochrome

FIGURE 1


NaIO4
R'-NH2
R'-C-NH-NH2
A
B
Schiff Base (Imine)
Hydrazone
NaBH3CN
Amine

In addition to the above-mentioned chemical advantages of using the hydrazide delivery system for labelling, these enzyme hydrazides have other practical applications:

(a) they have been introduced for enzyme immunoassay, for blotting, and for other histochemical and cytochemical purposes (15,19);

(b) enzyme hydrazides have been used for the microscopic analysis of cell surface glycoconjugates and as stains for the same glycoconjugates separated from extracts by SDS-PAGE and transferred to blots (19,20).

In our laboratory the glycoenzymes peroxidase and glucose oxidase conjugated with adipic dihydrazide have been used in a brief study by S. Boss (25) for immobilization on nylon tubing.

In this study, a systematic investigation of this method of protein-protein conjugation was undertaken. The two glycoenzymes were oxidized with periodate; one was reacted with excess adipic dihydrazide to form hydrazone, with free distal hydrazides and then it was conjugated to the other oxidized glycoenzyme as illustrated in Figure 2.

A. Enzymes Conjugated in This Study

The two enzymes investigated in this study, glucose oxidase and horseradish peroxidase, are glycoproteins of known carbohydrate content.

1. Glycoenzymes

A large number of enzymes contain covalently linked carbohydrate residues as part of their molecular structure. This group of glycoenzymes occur in a variety of biological materials including plant tissue, animal organs or microbial

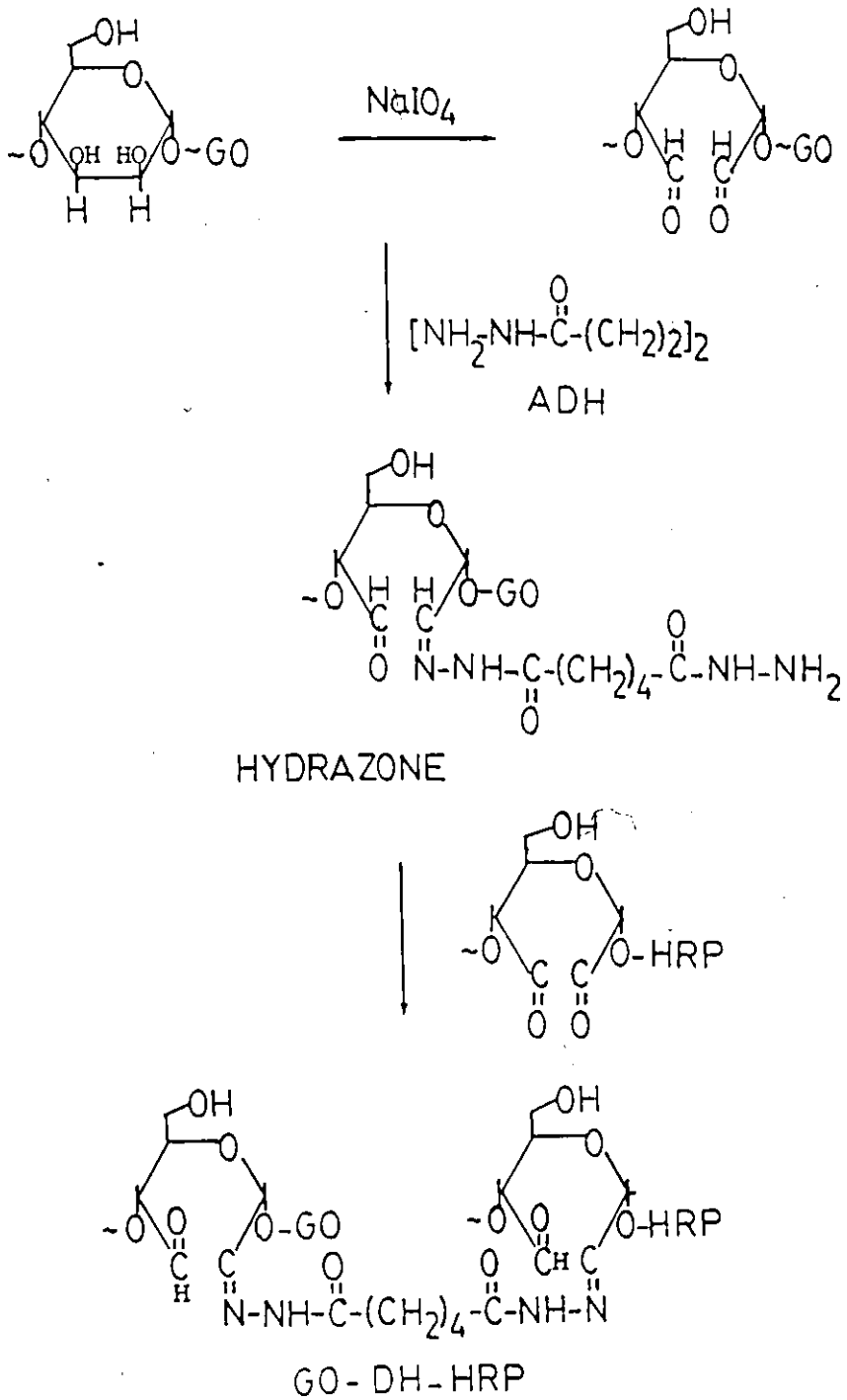
FIGURE 2

CONJUGATION OF PEROXIDASE AND GLUCOSE OXIDASE THROUGH THEIR CARBOHYDRATE MOIETIES USING ADIPIC DIHYDRAZIDE

Legend

Conjugation of the two glycoenzymes involves the site-specific modification of the oligosaccharide moieties. Mild periodate oxidation of both enzymes results in the generation of aldehyde groups which then condensed specifically with adipic-dihydrazide to form a hydrazone.

FIGURE 2



sources and may be soluble or membrane bound, extracellular or intracellular (26). The carbohydrate moiety is attached to the protein by an N-glycosyl link to the amide groups of asparagine or an O-glycosyl bond between the carbohydrate and the hydroxy function of serine, threonine or hydroxylysine (26,27).

Structurally, it has been found that glycoproteins contain only a few different monosaccharides and vary little in structural patterns but differ greatly in their total carbohydrate content, the number and distribution of these residues and in the degree of branching (27). As a rule, the oligosaccharide units of proteins containing N-glycosyl linkages possess a common core consisting of a pentasaccharide, trimannosyl-di-N-acetylglucosamine [$Man_3(GlcNAc)_2$] with further substitution of the peripheral mannose residues, either by mannose-rich oligosaccharides or more complex N-acetyllactosamine sialic acid derivatives (28).

The function of the carbohydrate residues in glycoproteins is of particular interest to researchers. It is apparent from the accumulated evidence that the catalytic activity of glycoenzymes is not in any way associated with their carbohydrate content (29). Glycoproteins are generally more resistant to proteolysis and denaturation than other proteins leading to the suggestion that the carbohydrate moiety is present in a protective capacity. Carbohydrate units attached to amino acids close to the surface may protect the three dimensional structure of the protein by sterically hindering interaction with and hydrolysis of the enzyme by proteolytic agents. Resistance to heat extremes may also be attributed to steric effects that prevent molecular transformations necessary for denaturation (27,28). That the carbohydrate may contribute to the antigenicity of the enzyme and could be involved in transport mechanisms have

also been postulated (29,30).

2. Glucose Oxidase

Glucose oxidase from *Aspergillus niger* is a flavoprotein consisting of two identical polypeptide subunits (mol. wt. 95,000) covalently linked by disulfide bonds. Each subunit of this globular protein contains one mole of iron and one mole of FAD (31-35). Although amino acid sequencing and crystallographic data are incomplete, the enzyme is known to contain 78% protein, 18% neutral sugar and 4% amino sugars (31,36).

Glucose oxidase is specific for β-D-glucose with the following overall reaction:

$$\text{Glucose} + O_2 \xrightarrow{GO} \text{Gluconic Acid} + H_2O_2$$

Enzymatic methods for glucose determination in biological fluids using glucose oxidase are numerous: a fluorometric method utilizes peroxidase and homovanillic acid (37); a polarographic oxygen sensor permits direct determination of glucose in the presence of glucose oxidase (38); photometric measurements of glucose are possible by peroxidase-coupled reaction to a chromogenic oxygen acceptor such as o-dianisidine, o-tolidine, or indophenol (39); another approach introduced by Trinder, involves the oxidative coupling of 4-aminoantipyrene (4-AAP), an auxochrome such as phenol or a phenol derivative and peroxidase to yield a red chromogen (40-42).

The work of Nakamura and Pazur has revealed that the carbohydrate moieties of glucose oxidase are not involved in its catalytic properties but act in a protective capacity at temperature extremes or in the presence of denaturing agents (28,32). Based on these observations, researchers (13) introduced the immobilization of the enzyme to an amino containing support, p-aminostyrene.

This approach was applied in our laboratory by S. Boss (25) by immobilizing the glucose oxidase to amino-containing nylon tubing, as well as its conjugation to peroxidase.

3. Peroxidase

Horseradish peroxidase is a globular glycoprotein (diameter 50Å) of molecular weight 40,000. The enzyme consists of 308 amino acid residues, a hemin group and eight neutral carbohydrate side chains attached through N-glycosyl linkages with asparagine (43,44). The carbohydrate moiety of this enzyme constitutes about 18% of the molecular weight.

Aibara et al. isolated 7 major isoenzymes from a crude extract of the plant enzyme; the most abundant is isoenzyme C (44). Peroxidase has been extensively studied with respect to structure, physical and catalytic properties and amino acid sequence (43-47). Attempts at X-ray crystallography have proven unsuccessful for the plant peroxidases however, recent work has led to the availability of this data for yeast cytochrome C peroxidase, a possible structural homologue of the plant enzyme (48,49). It should be noted that while the structural features common to both cytochrome C and horseradish peroxidases are remarkable some differences do arise. For example, unlike the yeast source, the plant peroxidase contains disulfide bonds and binds two moles of calcium per molecule. In addition, the plant peroxidases are glycoproteins and react with small aromatic hydrogen donors but not with cytochrome C (48,49).

From Poulos' and Welinder's work, prediction of the structural form and active site geometry of the plant enzyme has been introduced. A general model for peroxidase focusing on helices, disulfide-linkages and carbohydrate attachments as proposed by Welinder is illustrated in Figure 3A (49).

FIGURE 3

A STRUCTURAL MODEL OF HORSERADISH PEROXIDASE

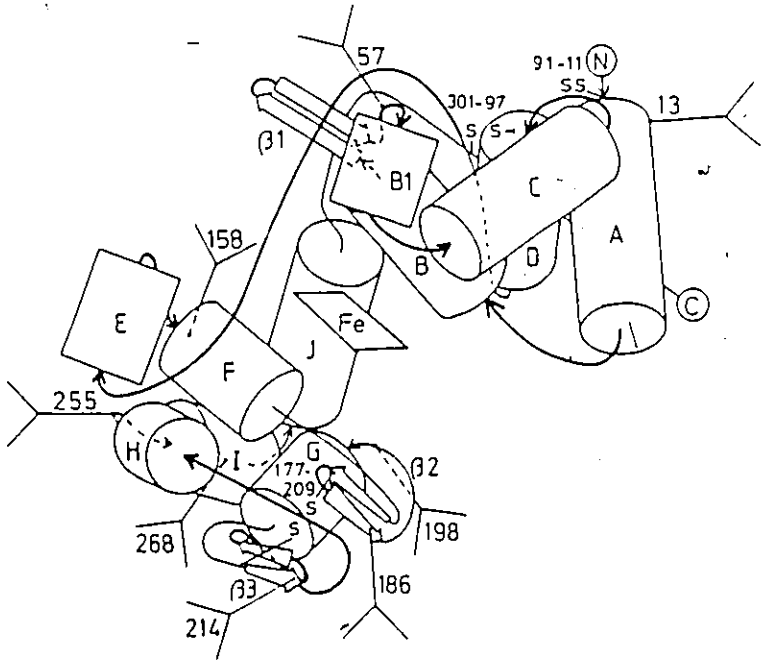
Legend

A. The structural model of peroxidase presented by Welinder (49) is illustrated. Cylindrical projections A-J represent helices of the enzyme. Extended structures β_1, β_2 and B_3 are indicated by arrows while the reverse turns and coils are shown as connecting lines. The amino and carboxy terminals are labelled as N and C, respectively. The three disulfide bridge residues of plant peroxidases are indicated by -SS- and the corresponding residue numbers. The hemin prosthetic group is indicated by the central rectangle, while the points of carbohydrate attachment are illustrated by a fork shaped symbol and numbers (49).

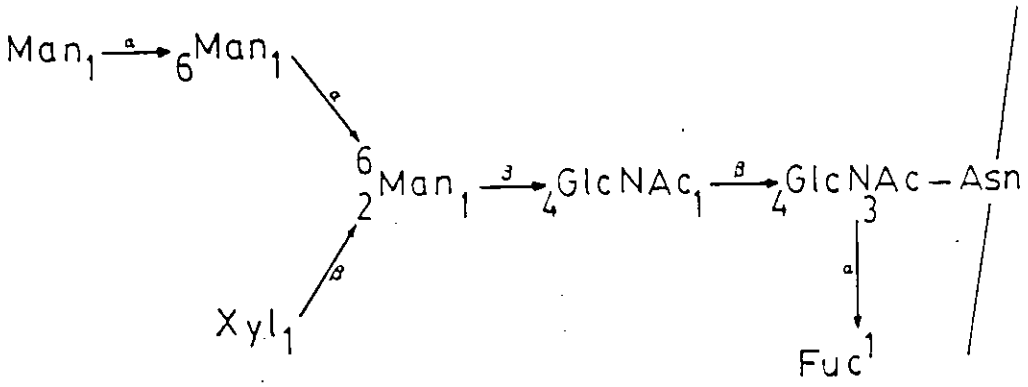
B. A schematic representation of the carbohydrate moieties of pineapple stem bromelain is indicated. As with the plant peroxidases, this unit of bromelain is linked to the protein through asparagine residues (50).

FIGURE 3

A


57
91-111
N
SS
301-97
S
S
13
β1
B1
C
A
158
B
D
C
E
Fe
J
F
255
H
I
G
177-
209
β2
S
198
268
S
β3
186
214

B


Man1 α 6Man1
α
6
2
Man1 3 4GlcNAc1 β 4GlcNAc – Asn
3
β
Xyl1
α
Fuc1

Although the carbohydrate chains have not been sequenced, they are known to be mannose-rich derivatives and because of their compositional similarity are believed to resemble those of another plant glycoprotein, pineapple stem bromelain (49-51). Figure 3B shows the 2 N-acetylglucosamine, 3 mannose glycan core found in bromelain.

Peroxidase is highly specific for the hydrogen acceptor, only H_2O_2, CH_3OOH and C_2H_5OOH of a number of compounds tested are active. Peroxidase may act as an oxidase with indirect electron transport to the heme by phenols or aromatic amines.

The overall reaction catalyzed by peroxidase is:

$$\text{Donor} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{oxidized Donor} + H_2O$$

Oxidation of the native enzyme's carbohydrate causes no decrease in activity (11). This observation led researchers to attempt conjugation of this enzyme through the carbohydrate moieties (11,12). One interesting development from this approach has been the preparation of peroxidase-antibody conjugates for enzyme immunoassay. Immobilization of this enzyme through its carbohydrate moiety on p-aminostyrene or Sepharose have been described (13,52). This approach of immobilizing HRP on a solid support was adopted in our laboratory previously (25,53).

B. Approaches Taken in This Study

The primary objective of this study was to prepare, characterize and optimize methods of covalent conjugation of horseradish peroxidase and glucose oxidase. The approach taken was shown previously in Figure 2 for GO and HRP. This is used as a model study which could be widely applied for conjugating any two glycoproteins through their carbohydrate moieties. Such conjugates could be useful in biochemistry, histochemistry and immunochemistry. The effect of using different low concentrations of periodate for oxidizing HRP and GO was investigated.

Evaluation of HRP and GO activities before and after conjugation was completed using the chromogen system of Artiss et al. illustrated in Figure 4 (41,42).

FIGURE 4

A SCHEMATIC REPRESENTATION OF THE ACTIVITY ASSAYS OF PEROXIDASE AND GLUCOSE OXIDASE

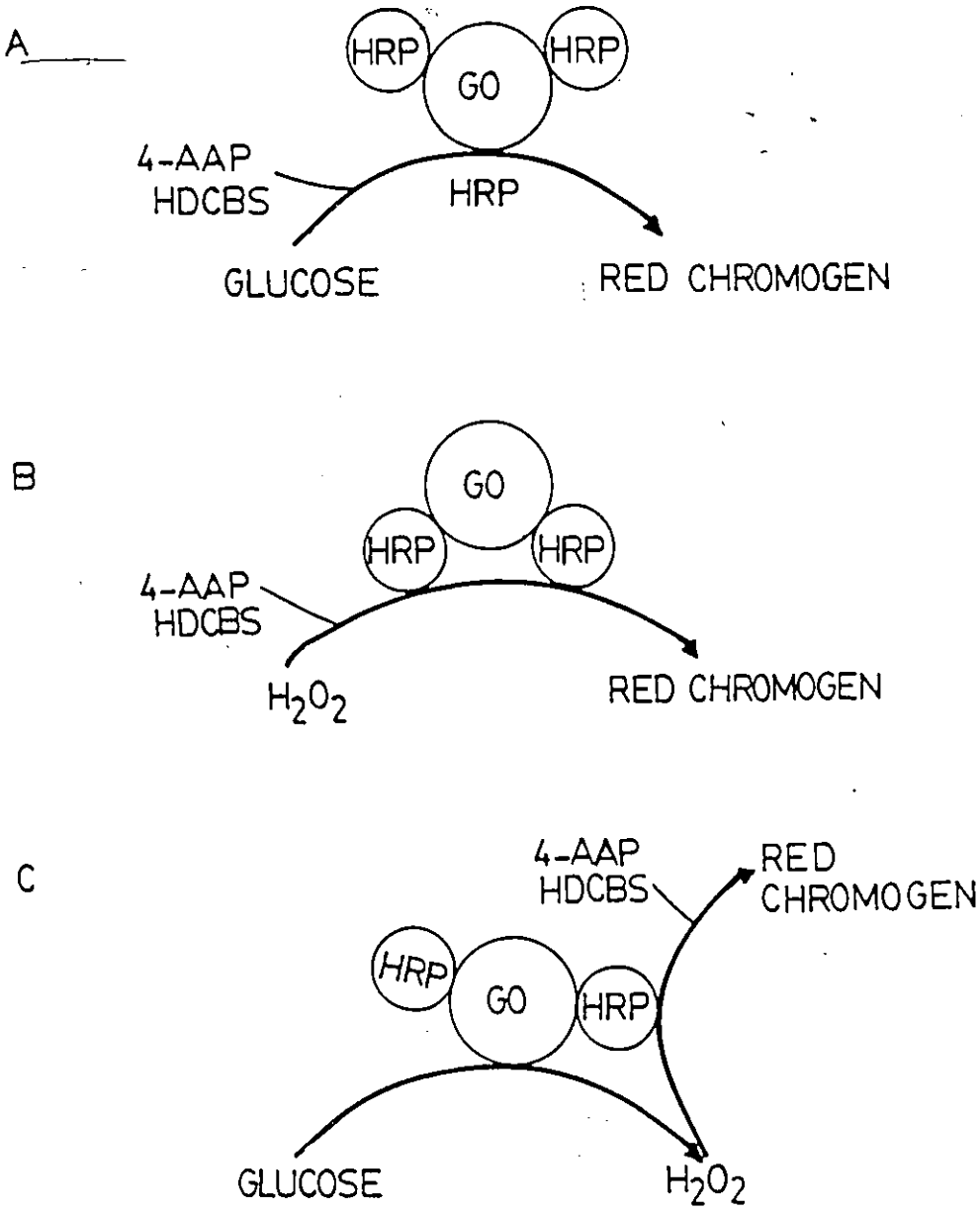
Legend

A. Conjugated glucose oxidase (GO) was assayed using the same method as the native enzyme by the peroxidase catalyzed coupling of 4-aminoantipyrene (4-AAP) and 2-hydroxy-3,5-dichlorobenzene sulfonate (HDCBS) with glucose as a substrate to form a red chromogen (41,42). Here additional free HRP is present in the mixture.

B. Conjugated peroxidase (HRP) was assayed employing the same chromogenic system, 4-AAP and HDCBS with peroxide as a substrate.

C. Activity of the conjugate (HRP + GO) was measured with glucose as a substrate using the modified Trinder reaction described above for peroxidase and glucose oxidase. In this case no free HRP was present.

FIGURE 4


A
HRP
GO
HRP
4-AAP
HDCBS
HRP
GLUCOSE
RED CHROMOGEN
B
GO
HRP
HRP
4-AAP
HDCBS
H_2O_2
RED CHROMOGEN
C
4-AAP
HDCBS
RED
CHROMOGEN
HRP
GO
HRP
GLUCOSE
H_2O_2

CHAPTER II

EXPERIMENTAL

A. Materials

Glucose oxidase (*Aspergillus niger*) [β-D-Glucose: oxygen 1-oxidoreductase, E.C. 1.1.34] Type X-S was purchased from Sigma Chemical Company, St. Louis, MO 63178.

Peroxidase (Horseradish [Donor: Hydrogen peroxide oxidoreductase; E.C. 1.11.1.7] Grades I,II (for enzyme immunoassay) were obtained from Boehringer-Mannheim Canada, Dorval, PQ H9P 1A9.

The enzyme activities quoted are those of the supplier. Unit definitions are as follows: GO, one unit is the amount of enzyme that will oxidize one μmole of β-D-glucose to D-gluconic acid and H_2O_2 per minute at pH 5.1 at 35°C; HRP, one unit is the amount that will catalyze the oxidation of one μmole of guaiacol by H_2O_2 per minute at 25°C pH 7.5.

Amicon Corporation Scientific System Division, Danvers, MA, 01923 was the source of the ultrafiltration membranes YM-30, YM-100 and XM-300 used throughout the study.

The analytical grade reagents purchased from Aldrich Chemical Co., Milwaukee, WI 52322 were: adipic dihydrazide, 4-aminoantipyrene and sodium 2-hydroxy-3,5-dichlorobenzenesulfonate.

Sodium periodate was obtained from BDH Chemical, Toronto, ON M8Z 1K5.

2,4,6-Trinitrobenzenesulfonic acid was purchased from Eastman Kodak Co., Rochester, NY 14650.

The following reagents were purchased from Fisher Scientific Co.: Standard buffer solutions, ethylene glycol, D-glucose and hydrogen peroxide (30% w/v).

B. Instrumentation

For the purification of the oxidized and conjugated enzyme preparation an Amicon ultrafiltration cell (10 mL volume) was employed. An operating pressure of 4 atm nitrogen was maintained throughout the procedure. Membranes were soaked in distilled water for one hour and pre-conditioned with 1 mg/mL bovine serum albumin before use. For reuse, membranes were stored in 10% ethanol at $4^{o}C$.

All spectrophotometric measurements and recordings, activity assay and kinetic measurements were completed on the 8451A Diode Array Spectrophotometer with 7470 plotter from Hewlett Packard, Palo Alto, CA 94304.

The micropipettors used in this study were Gilson Models P-200 D and P-1000 D with disposable pipet tips available from Mandel Scientific Company Ltd., Rockwood, ON N0B 2K0.

For weights above one gram, a Mettler PC 4400 Delta Range electronic balance was used. For weights below one gram, a Mettler H1b semi-automatic balance, Fisher Scientific Co., was employed.

Verification of pH measurements were completed on a Fisher Accumet pH meter, Model 800.

C. Reagents

All aqueous solutions were prepared using deionized distilled water. The following solutions were used:

2,4,6-Trinitrobenzenesulfonic acid (TNBS) solution: 0.01 M; This solution was stable for one week in the dark at 4°C. Bicarbonate Buffer: 0.05 M, pH 9.2; Phosphate Buffer: 0.1 M, pH 7.5; Borate Buffer: 0.05 M, pH 9.2; Sodium periodate stock solutions of 8, 12 and 20 mM were prepared immediately before use. Adipic dihydrazide stock solution of 20 mM was prepared in 0.1 M phosphate buffer at pH 7.5. Chromogen stock solutions: 2-hydroxy-3,5-dichlorobenzenesulfonate (HDCBS): A solution with a concentration 18 mM HDCBS in 0.1 M phosphate buffer, pH 7.5 was prepared. This solution is stable for one week when stored in the dark at 4°C. 4-AAP: This solution was prepared to contain 8 mM 4-aminoantipyrene in 0.1 M phosphate buffer pH 7.5. It is stable for two weeks when stored in the dark at 4°C. Stock Hydrogen Peroxide: 10 mM stock solution was prepared fresh in distilled water immediately before the experiment. Glucose stock solution: A stock solution of 1 M D-glucose in distilled water was permitted to mutarotate 24 h prior to use. Peroxidase Solution: 150 x 10^3 U/L stock solution was prepared in 0.1 M phosphate buffer pH 7.5. For the determination of glucose oxidase activity 20 µL of this stock was used to give 3 units in a 1-mL cuvette.

D. Methods

1. Performance of HRP Assay

Rate method: The reaction was measured at room temperature in 0.1 M phosphate buffer pH 7.5. The sample and blank were always prepared fresh to contain 2.4 mM 4-AAP, 9 mM HDCBS and 100 µL of peroxidase solution in 1 mL. Reaction was initiated by adding 100 µL of 1 mM peroxide to the sample cell and 100 µL of distilled water to the blank (reference cell). The change in absorbance at 510 nm was continuously monitored in a 1-cm pathlength cell for 2 minutes.

2. GO Assay

The initial rate of reaction was conducted as for the HRP assay, so that the sample and blank were prepared to contain 2.4 mM 4-AAP, 9 mM HDCBS, 80 μL of glucose oxidase solution and 20 μL of peroxidase solution to give 3U of the latter in a 1-cm pathlength cell. The reaction was initiated by adding 100 μL of 1 M glucose to the sample cell and 100 μL of distilled water to the reference cell. The reaction was monitored for 2 minutes at 510 nm.

3. GO-Dihydrazide-HRP Conjugate

Oxidation: A fresh solution containing 2 mg/mL GO or HRP was made in 0.1 M phosphate buffer pH 7.5. The initial concentration of enzyme was calculated spectrophotometrically using an extinction of 28,200 $M^{-1}cm^{-1}$ at 450 nm for glucose oxidase and ε = 102,000 $M^{-1}cm^{-1}$ at 404 nm for peroxidase (34,54). In the conjugation procedure initial and final concentrations of HRP and GO were determined by a multicomponent analysis available as software on the Hewlett Packard 8451 Spectrophotometer. This analysis is based on the spectral differences of the two enzymes.

Oxidation of the enzyme was initiated by mixing 1 mL of 8 mM, 12 mM or 20 mM stock sodium metaperiodate and 1 mL of enzyme. The reaction mixture was incubated for 2 h at room temperature (RT) in the dark. Excess periodate was destroyed with the addition of 1 mL of 0.32 M ethylene glycol and incubation for 20 minutes at RT in the dark.

The resulting oxidized GO or HRP was isolated by ultrafiltration using an Amicon YM-100 ultrafiltration membrane for glucose oxidase and a YM-30 membrane for HRP. The enzyme was thus washed with two 10-mL volumes of coupling buffer before removing it from the Amicon cell. Concentrations of the

oxidized enzymes were checked again by spectrophotometric analysis, and an activity test on each enzyme was conducted.

Reaction with Adipic Dihydrazide (ADH): Oxidized GO or HRP was incubated with a solution containing a final concentration of 10 mM ADH in coupling buffer pH 7.5 for 3h at RT. Excess reagent was removed by ultrafiltration using YM-100 membrane for GO-DH and YM-30 membrane for HRP-DH. Concentration of the enzyme was verified by spectrophotometric analysis, an activity test was conducted and a TNBS titration to measure the amount of hydrazido group conjugated to the enzyme was performed.

GO-DH-HRP Conjugate: Oxidized HRP, prepared as for oxidized GO, was permitted to conjugate with the dihydrazide modified GO in a ratio of 7 to 1 overnight at 4°C. Free HRP and GO-DH were removed by ultrafiltration with YM-100 and XM-300, respectively. The amount of GO-DH-HRP conjugate collected was subjected to multicomponent analysis, and activity tests on each enzyme and the conjugate were performed.

4. Determination of Hydrazido Groups Incorporated Into Oxidized Glucose Oxidase Using the TNBS Test

The TNBS test was performed by modifying the Snyder et al. procedure (55). The test was used in this work to determine the amount of dihydrazide reacted with oxidized GO or oxidized HRP. 100 µL of GO-DH solution 0.1 M phosphate buffer at pH 7.5 was made up to 900 µL by the addition of 800 µL of the same buffer. The reaction was initiated by the addition of 100 µL of 0.01 M TNBS solution. A reference sample was prepared at the same time to contain 100 µL of 0.01 M TNBS and 900 µL of the above buffer. The reaction mixture was monitored from zero time to one hour at 500 nm. In this experiment,

the zero time absorption was taken as a solution of the same concentration of GO-DH as the reaction mixture but without TNBS. The absorption at 500 nm of the reaction mixture and zero time was subtracted and then the difference was divided by the extinction coefficient of one hydrazido group of adipic dihydrazide carried through the same reaction, $\epsilon = 15{,}000\ M^{-1}cm^{-1}$.

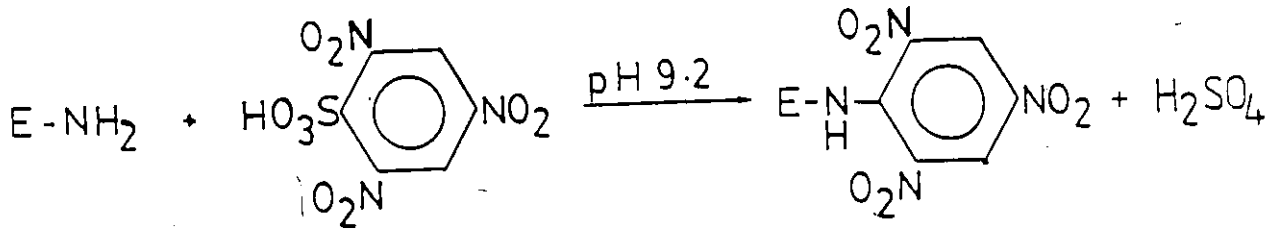
CHAPTER III

RESULTS AND DISCUSSION

A. Distinguishing Amino and Hydrazido Groups on the Glycoenzyme

The oxidized glycoenzyme was reacted with excess adipic dihydrazide (ADH) to form a hydrazone with a distal free hydrazide. The amino and hydrazido groups on this modified protein were distinguished by using a TNBS titration (Scheme 1).

For the amount of hydrazido groups reacted with the oxidized enzyme, the TNBS test was carried in 0.1 M phosphate buffer pH 7.5, the maximum absorbance was at 500 nm (53). For e-amino groups on native enzyme the same test was carried in 0.05 M bicarbonate buffer pH 9.2 with maximum absorbance at 422 nm (56-58). Using a standard curve (Figure 5), based on ethanolamine as a model for the e-amino group of lysine residues, the TNBS test was carried on native HRP. A total of 4.5 amino groups per molecule of enzyme were thus estimated which may be compared with the 6 lysyl residues reported in the enzyme's sequence (45).



Scheme 1

FIGURE 5

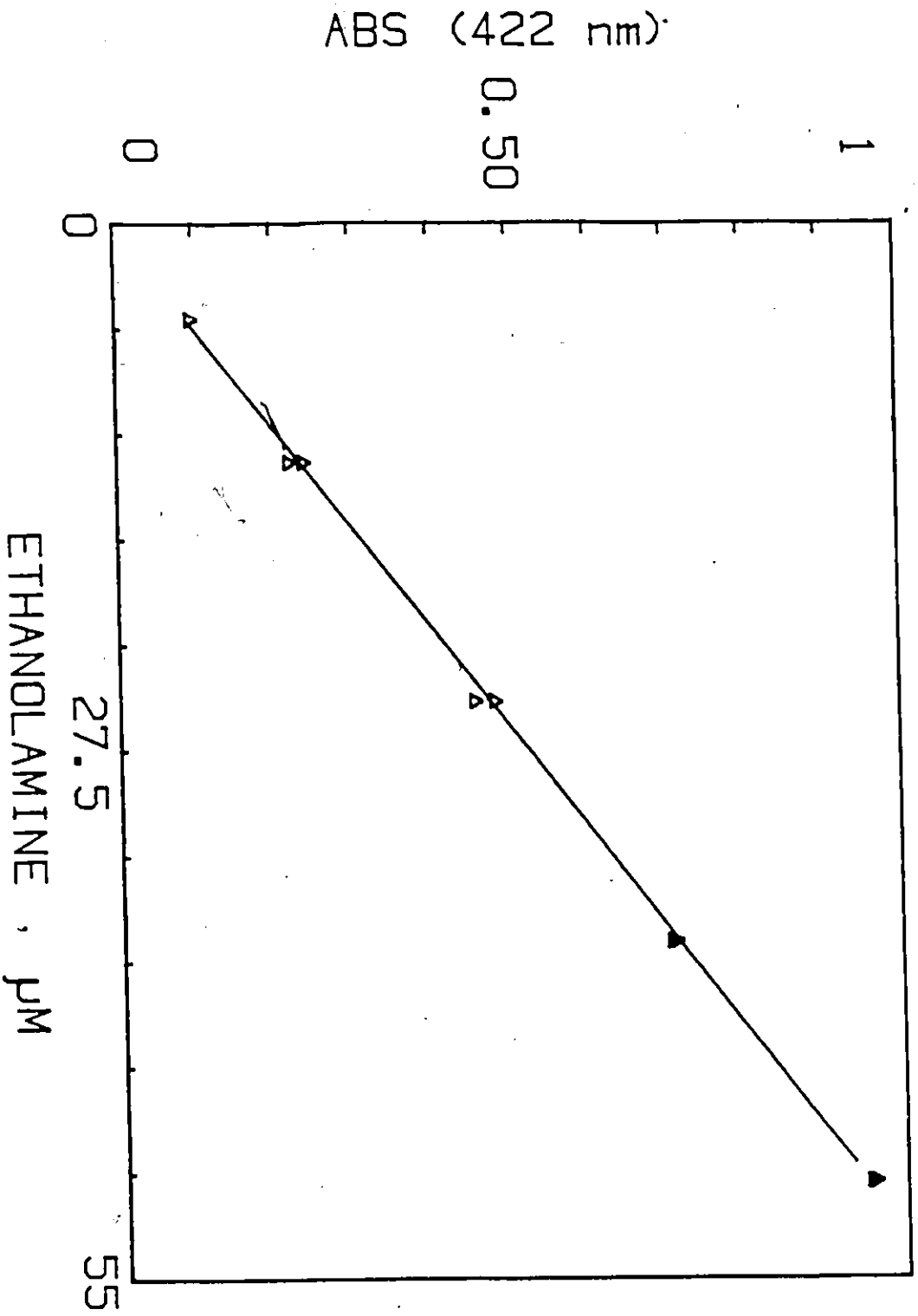
STANDARD CURVE FOR ETHANOLAMINE

Legend

Absorbance at 422 nm versus ethanolamine concentration between 5 and 50 μM, reaction was carried out in 0.05 M bicarbonate buffer pH 9.2 and 1 mM TNBS in a 1 cm pathlength cell. Readings were taken after 1-h incubation at RT.

Linear regression analysis of this data indicated a slope of 0.01910 ± 0.00019 $\mu M^{-1}cm^{-1}$, Y-intercept of -0.0042 ± 0.0032 and a correlation coefficient of 0.9995.

FIGURE 5



Ethanolamine was chosen as standard simply for expedience, but it is noted that the extinction coefficient determined here (Figure 5) is 19,100 $M^{-1}cm^{-1}$ which is very similar to that reported (56) for N-α-acetyl-L-lysine amide (19,150 $M^{-1}cm^{-1}$) as standard.

The TNBS test for ADH showed a linear relationship between ADH concentration and the color formation measured at the maximum of 500 nm, (Figure 6). Scheme II, shows the reaction of ADH with TNBS. The extinction coefficient determined here, 30,100 $M^{-1}cm^{-1}$ is for the bifunctional molecule. Half that value was used for TNBS titrations of exposed hydrazido groups incorporated into glycoproteins (see below).

B. Enzyme Activity

The assay sequence employed in the calculation of peroxidase and glucose oxidase activities involved the oxidative coupling of HDCBS and 4-AAP (41,42). This scheme has been extensively applied in the quantitation of peroxide in peroxide generating reactions. B. Dulay working in this laboratory, further extended the application of this indicator reaction to the assay of peroxidase activities (53), and this was further adopted by S. Boss (25). A schematic of the concept involved in the determination of conjugated glucose oxidase and peroxidase has been given earlier in (Figure 4).

1. Peroxidase Assay

Experiments conducted by B. Dulay showed that H_2O_2 concentrations greater than 0.3 mM have an inhibitory effect on peroxidase activity (53). These observations were also corroborated by S. Boss (25). The optimal hydrogen peroxide concentration for the assay was reinvestigated by this work. It was found that 0.3 mM H_2O_2 inhibited the enzyme activity by 25%, and that 0.1 mM was

FIGURE 6

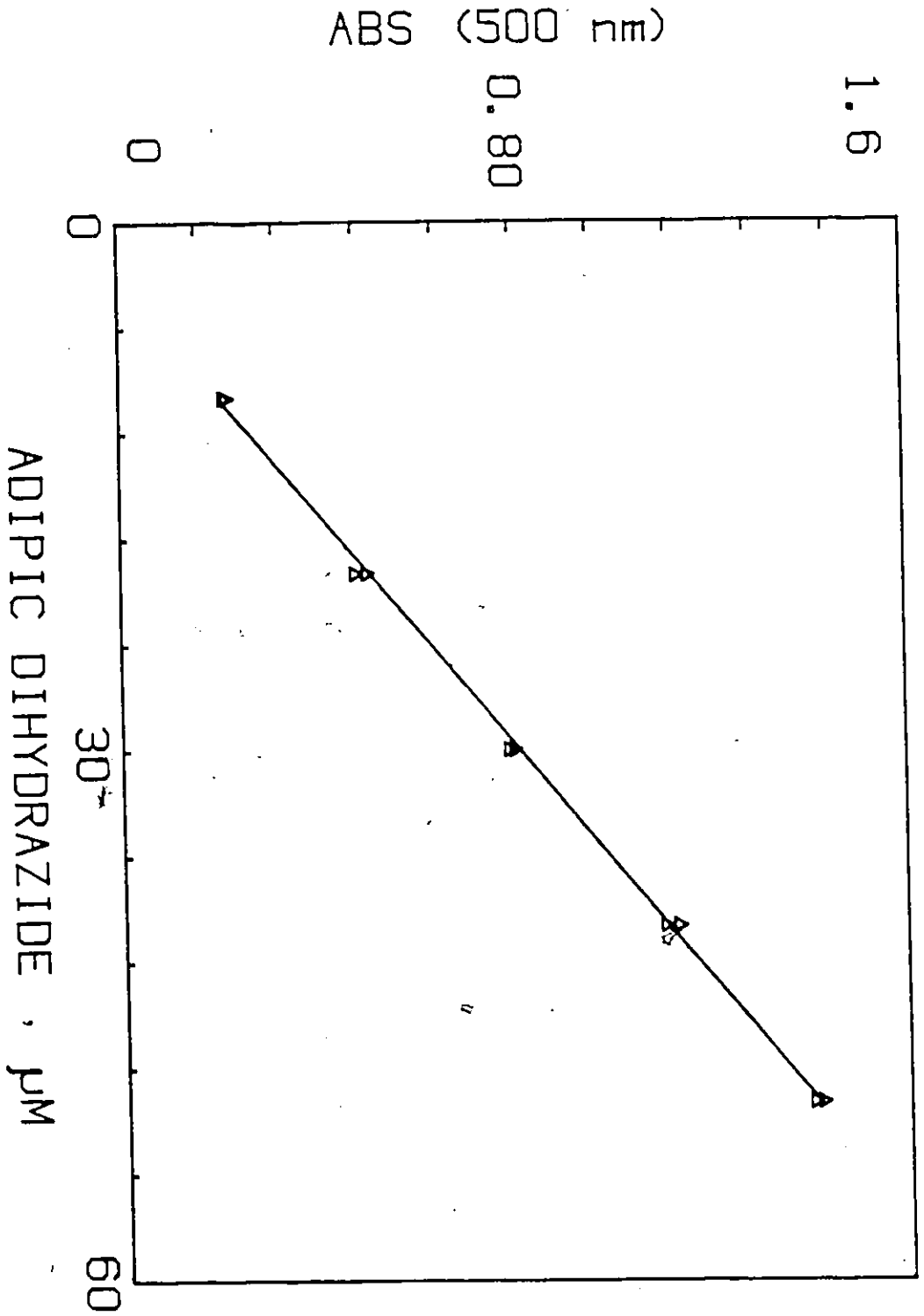
ADH STANDARD CURVE

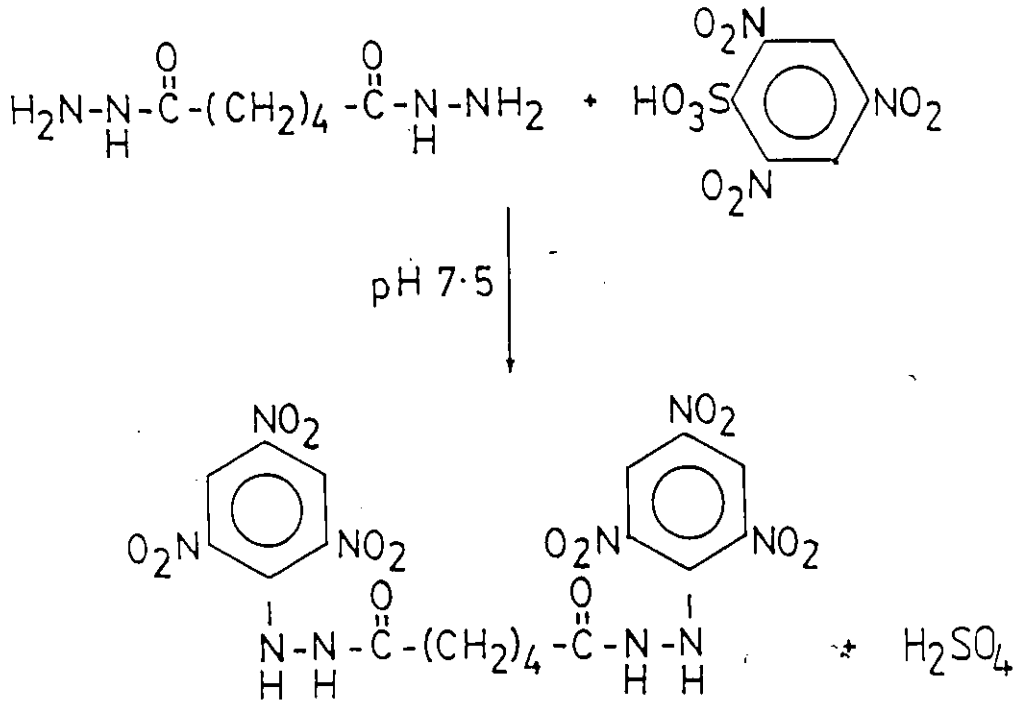
Legend

Absorbance at 500 nm versus ADH concentration between 10 to 50 μM, reaction carried out in 0.1 M phosphate buffer pH 7.5 and 1 mM TNBS in a 1 cm pathlength cell. Readings were taken after 1-h incubation at RT.

Linear regression analysis of this data indicated a slope of 0.0301 ± 0.0003 $\mu M^{-1}cm^{-1}$, Y-intercept of -0.0939 ± 0.0054 and a correlation coefficient of 0.9994.

FIGURE 6





Scheme 2

the optimal substrate concentration for kinetic assay (Table I). Using 0.1 mM H_2O_2, 9.0 mM HDCBS and 2.4 mM 4-AAP a linear relationship between the rate of color formation and HRP concentration can be realized (Figure 7). Peroxidase activities (U/L) were routinely calculated using the extinction coefficient derived from the standard curve for this chromogenic system (Figure 8).

2. Glucose Oxidase Assay

This assay was an adaptation of the peroxidase reaction for measurement of glucose oxidase activity. Here glucose, at 100 mM, was the substrate and the H_2O_2 produced from it by the glucose oxidase reaction was consumed immediately in the presence of 9.0 mM HDCBS, 2.4 mM-4AAP and 3U mL^{-1} soluble HRP as described by Artiss et al. (41,42). Under these conditions, variation of GO concentrations from 0.5 to 4.5 nM indicated a linear correlation between rate of absorbance change at 510 nm and concentration (Figure 9).

C. Effect of Oxidation on Peroxidase and Glucose Oxidase

The periodate reaction was chosen because of absence of side reactions, its high yield of aldehyde groups, and the fact that it can be carried out in aqueous solution at or near neutral pH. Nakane et al. (11) mentioned that other oxidizing agents such as lead tetraacetate which require acidic media were to be avoided since such media destroy HRP activity. Tijssen and Kurstak (12) reported that oxidation sensitivity of the carbohydrate moiety is a central problem. In their opinion, HRP is sensitive to impurities in water such as bacteria, bacteriostatic agents, polystyrene, etc. Yamasaki et al. (59) mentioned another possible side-effect of sodium periodate treatment, the oxidation of some amino acids which may alter the conformation of protein molecules. O'Shannessy et al. (60) reported that the oxidation of amino acids with periodate appears only

TABLE I

OPTIMIZATION OF HYDROGEN PEROXIDE CONCENTRATION[a]

H_2O_2 (μM)	ΔABS/min (510 nm)
50	0.713 ± 0.016[b]
70	0.737 ± 0.007
100	0.738 ± 0.003
200	0.636 ± 0.004
300	0.559 ± 0.004

(a) Rate of reaction was measured in the presence of 2.4 mM 4 AAP and 9 mM HDCBS with a 1 cm pathlength cuvette, varying the concentration of H_2O_2 as shown above at 25°C, in 0.1 M phosphate buffer pH 7.5.

(b) Results are the average of triplicate assays.

FIGURE 7

PEROXIDASE STANDARD CURVE

Legend

Assay of peroxidase activity was carried out in duplicate using 0.1 mM hydrogen peroxide with 9.0 mM HDCBS and 2.4 mM 4-AAP. Initial rates at room temperature were recorded at 510 nm.

Linear regression analysis of this relationship indicates a slope of 0.1755 ± 0.0015 $nM^{-1}min^{-1}$, Y-intercept of 0.0347 ± 0.0087 and a correlation coefficient of 0.9998.

Concentrations of HRP were determined by spectrophotometric analysis at 404 nm, $\epsilon = 1.02 \times 10^5\ M^{-1}cm^{-1}$.

FIGURE 7

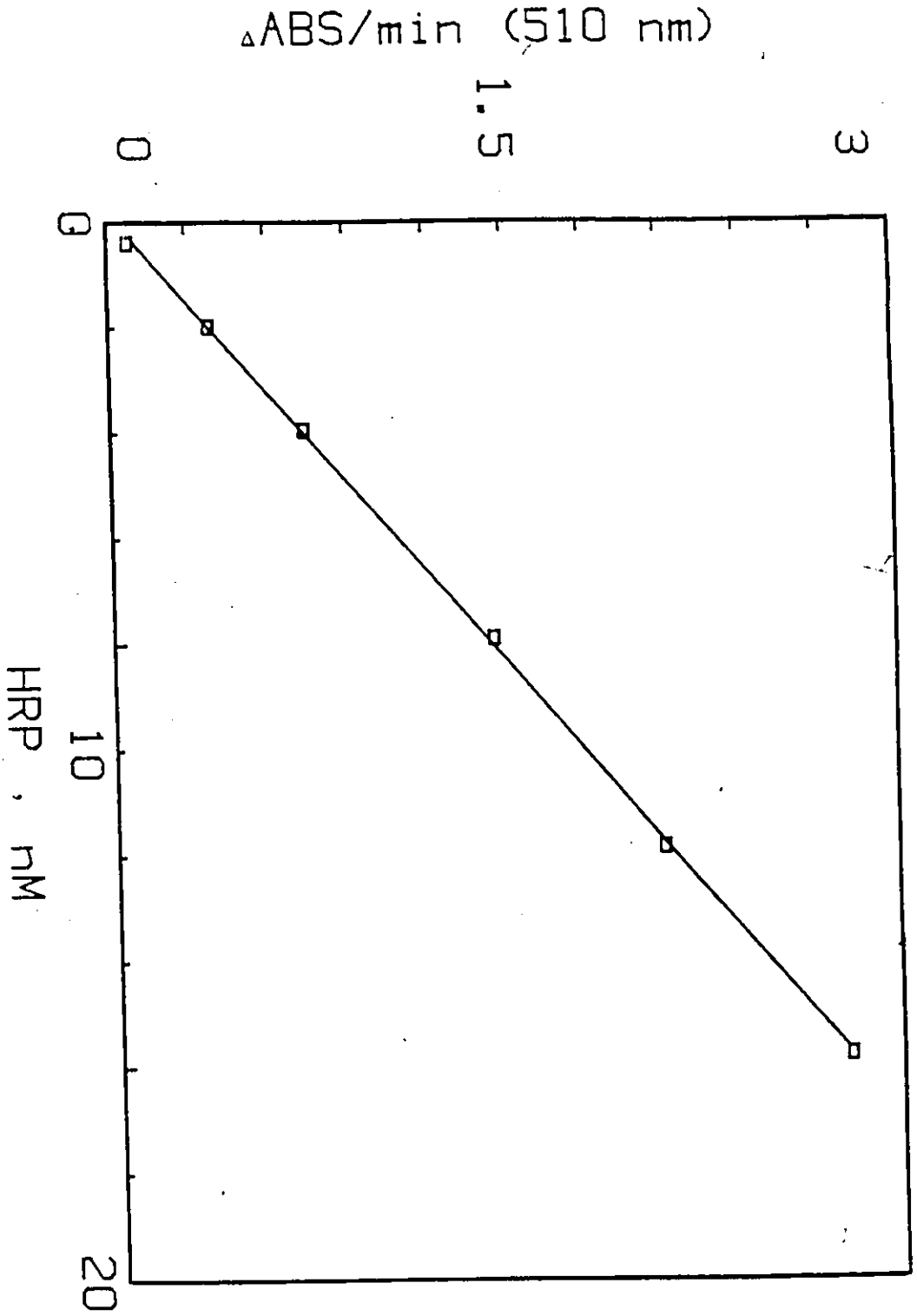


FIGURE 8

STANDARD CURVE FOR H_2O_2

Legend

The assay was carried out in duplicate at pH 7.5 in a 1-cm pathlength cuvet, using H_2O_2 concentrations between 2 and 30 μM, 9.0 mM HDCBS, 2.4 mM 4-AAP and 1 unit of HRP. Absorbance of the end point reaction at 3 min was recorded at 510 nm using HP spectrophotometer.

Linear regression analysis indicated a slope of 0.03376 ± (5.06×10^{-5}) $\mu M^{-1}cm^{-1}$, Y-intercept of $-0.00217 \pm 4.71 \times 10^{-4}$ and a correlation coefficient of 0.9999.

FIGURE 8

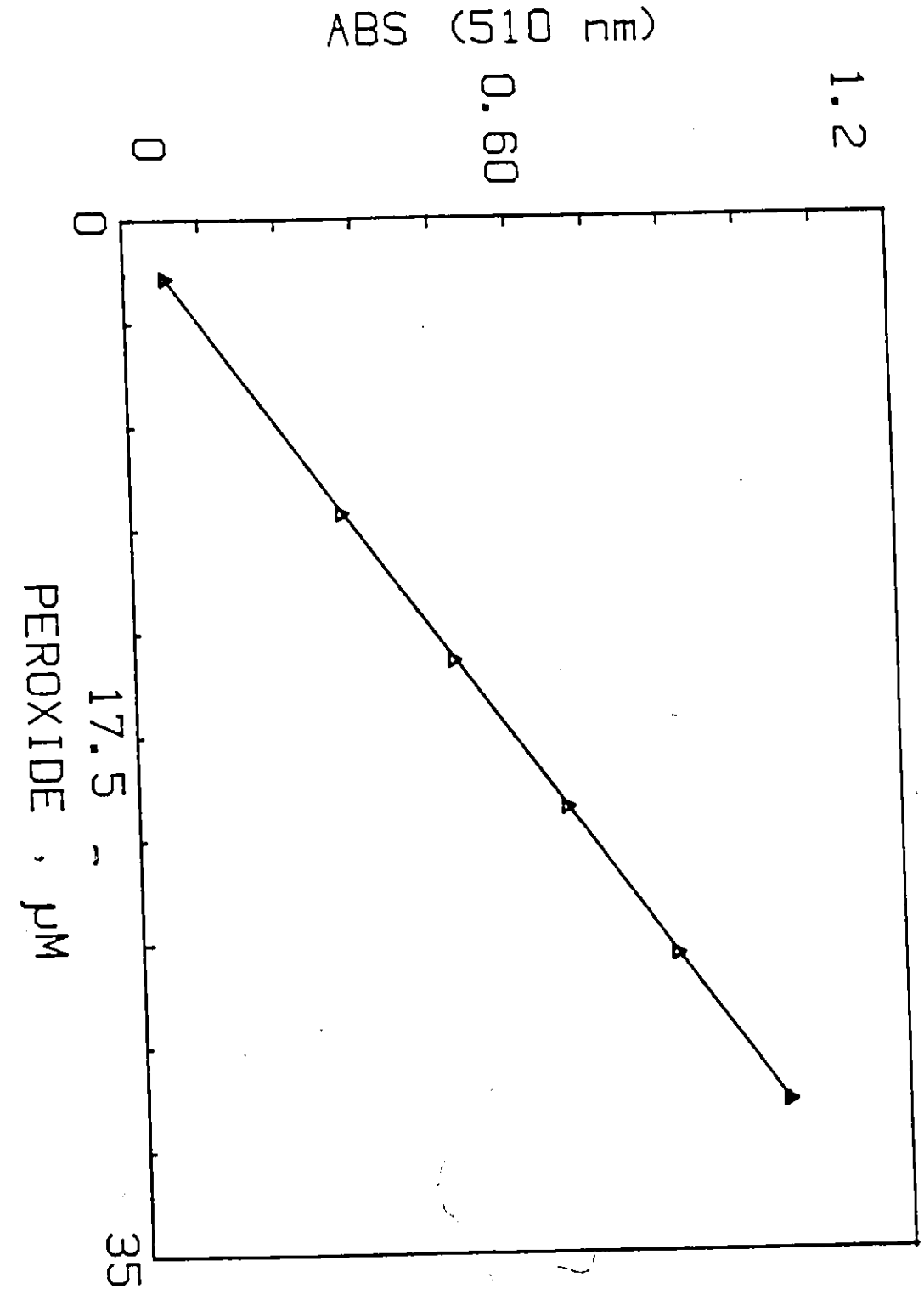


FIGURE 9

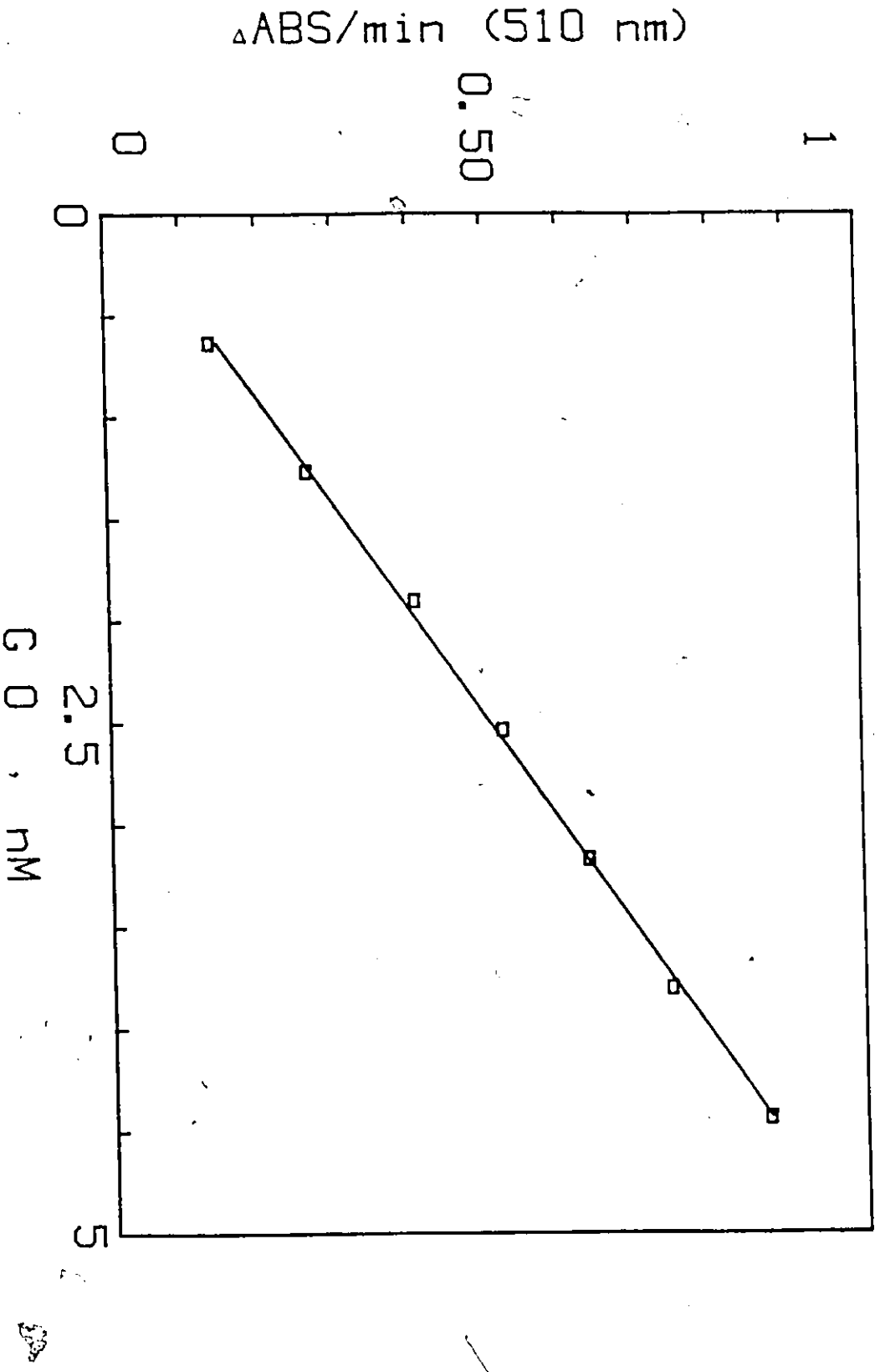
STANDARD CURVE FOR GLUCOSE OXIDASE

Legend

The assay was conducted on duplicate aliquots of GO employing the enzyme assay conditions described in the methods section. Initial rates were calculated from growth curves using 100 mM glucose. Concentrations of glucose oxidase were determined spectrophotometrically using, ϵ = 28,200 $M^{-1}cm^{-1}$ λ_{max} = 450 nm.

Linear regression analysis gave the following characteristics: slope 0.1902 ± 0.0032 $nM^{-1}min^{-1}$, Y-intercept = 0.0275 ± 0.0040, and a correlation coefficient of 0.9993.

FIGURE 9



to be a problem under conditions of extensive oxidation i.e., room temperature for several hours, and a very high concentration of periodate. In addition, carboxyl groups may be produced instead of the desired aldehydic functionalities. The conditions necessary for optimal periodate oxidation of HRP were investigated by Kurstak et al. (12), and later by B. Dulay in this laboratory (53). In their investigations, 4 to 8 mM was found to be the optimum concentration range.

A solution of glucose oxidase was subjected to oxidation with periodate at 4 to 10 mM for 2 hours. The data presented in Table II show small losses in relative specific activity and the turnover number of glucose oxidase.

A similar investigation was undertaken to evaluate the effect of periodate oxidation on the activity of peroxidase. The results of Table III show a relative specific activity and k_{cat} of oxidized peroxidase with little loss of enzyme activity.

D. The Effect of Hydrazide Incorporation on Peroxidase and Glucose Oxidase Activities

ADH was chosen to conjugate the oxidized glycoproteins because hydrazido groups offer several advantages over simple amino compounds (9,60). Firstly, the pKa of a hydrazide is approximately 2.6, whereas that of an amine is 8-9. Therefore, the use of hydrazide allows for the labelling reaction to be carried out under conditions that prevent the conjugation of the ε-amino groups on GO-DH derivative to the aldehyde functionalities on the modified HRP, that is at around neutral pH. Secondly, in neutral and acidic pH, the reaction product of a hydrazide and an aldehyde, a hydrazone linkage, is stable and does not require reduction with cyanoborohydride thereby circumventing one of the

TABLE II

THE EFFECT OF PERIODATE OXIDATION AND ADIPIC DIHYDRAZIDE REACTION ON GLUCOSE OXIDASE ACTIVITY

Preparation Number	$NaIO_4$[a] mM	Hydrazide/GO[b][c]	k_{cat}[d] before oxidation min^{-1}	k_{cat}[d] after oxidation min^{-1}	k_{cat}[d] after conjugation with ADH min^{-1}
1	4	12	12755	10,000 (78)[e]	8317 (65)
2	4	17	13761	12467 (87)	11000 (79)
3	6	31	14071	12275 (87)	11105 (79)
4	10	14	11887	9337 (79)	8170 (69)

(a) For different periodate concentrations, the oxidation was carried for 2 hours at room temperature on a 2 mg/mL solution of glucose oxidase in 0.1 M phosphate buffer pH 7.5.

(b) The concentration of ADH conjugated to the GO was determined using 1 mM of TNBS, incubated in dark for 1 h, reading was taken at 500 nm, ϵ = 15,000 $M^{-1}cm^{-1}$.

(c) The concentration of GO was determined at λ = 450 nm, ϵ = 28,200, (34) $M^{-1}cm^{-1}$.

(d) k_{cat} was calculated using the maximum activity of the enzyme in (U) divided by the enzyme concentration. Assays were performed in triplicate.

(e) number in brackets is the relative specific activity of the enzyme as a percentage of that of the native enzyme.

TABLE III

THE EFFECT OF PERIODATE OXIDATION AND ADIPIC DIHYDRAZIDE TREATMENT ON PEROXIDASE ACTIVITY

Preparation Number	$NaIO_4$[a] mM	Hydrazide[b] HRP[c]	k_{cat}[d] before oxidation min^{-1}	k_{cat}[d] after oxidation min^{-1}	k_{cat}[d] after conjugation with ADH min^{-1}
1	4	10	5881	5423 (92)[e]	3776 (64)
2	4	5	5958	4559 (77)	3800 (64)
3	6	8	4692	3950 (84)	3257 (69)
4	10	8	5780	5200 (90)	4000 (69)

(a) For different periodate concentration, the oxidation was carried for 2 h at room temperature on a 2 mg/mL solution of peroxidase in 0.1 M phosphate buffer pH 7.5.

(b) The concentration of adipic dihydrazide conjugated to HRP was determined using 1 mM of TNBS, incubated in the dark for 1 h reading was taken at 500 nm ϵ = 15,000 $M^{-1}cm^{-1}$.

(c) The concentration of peroxidase was determined spectrophotometrically at λ = 404 nm, ϵ = 102,000 $M^{-1}cm^{-1}$ (54).

(d) k_{cat} was calculated using the ratio of the maximum enzyme activity (Units) to the enzyme concentration. Assays were performed in triplicate.

(e) Number in brackets is the relative specific activity of the enzyme as a percentage that of the native enzyme.

reactions associated with labelling with amino compounds. On that basis the reaction of ADH with oxidized GO and/or HRP was carried for 3 h, after which the free ADH was eliminated using YM 100 and YM 30 Amicon membranes for GO-DH and HRP-DH, respectively. The amount of ADH incorporated into oxidized enzyme in a 3-h incubation, was the same as for 5 h and overnight incubations. The molar ratio of dihydrazide conjugated to GO was calculated, assuming mono-attachment, and is given with the activity tests of GO in Table II. On average, 73% of GO specific activity was maintained.

The same experiment was carried with oxidized peroxidase. The molar ratio of dihydrazide conjugated to the enzyme was calculated as mentioned above; results are shown in Table III. Thirty to 37% of peroxidase activity was lost compared to the activity of native enzyme.

The small loss of enzyme activity after each treatment was accompanied by a decrease in the yield compared to the native enzyme. Glucose oxidase, Table IV, showed a slight decrease in the yield after oxidation, and further loss after reaction with dihydrazide. Overall 38 to 50% recovery was obtained by this two-step procedure.

Better overall yields were obtained for modified peroxidase compared to the native enzyme (Table V). The minor loss after oxidation was followed by a greater loss in yield after dihydrazide treatment, with overall recoveries being 56 to 60%. The decrease in yield was accompanied by a decrease in RZ value ($\lambda_{404}/\lambda_{278}$) from 3.2 for the native HRP to 2.25 for peroxidase-hydrazide. The decrease in RZ resulted primarily from the relative increase in absorption at 278 nm. However, no shift was observed in the Soret-band (λ_{404} nm) for oxidized or dihydrazide treated peroxidase.

TABLE IV

PERCENT[a] OF GLUCOSE OXIDASE RECOVERED AFTER OXIDATION AND DIHYDRAZIDE TREATMENT AT DIFFERENT CONCENTRATIONS OF PERIODATE

$NaIO_4$ mM	Yield after oxidation (%) compared to original	Yield after dihydrazide treatment (%) compared to original
4	90 ± 2	50 ± 6
6	92 ± 4	38 ± 3
10	90 ± 3	44 ± 3

(a) Each percent is an average of triplicate preparations, based on the concentration of GO which was determined spectrophotometrically at λ = 450 nm, ϵ = 28,200 $M^{-1}cm^{-1}$.

TABLE V

PERCENT[a] OF PEROXIDASE RECOVERED AFTER OXIDATION AND DIHYDRAZIDE TREATMENT AT DIFFERENT PERIODATE CONCENTRATIONS

$NaIO_4$ mM	Yield after oxidation (%) compared to original (RZ)[b]	Yield after dihydrazide treatment (%) compared to original (RZ)
4	84 ± 2 (2.90)	56 ± 3 (2.20)
6	90 ± 2 (2.80)	57 ± 8 (2.35)
10	86 ± 3 (2.75)	60 ± 3 (2.25)

(a) Each percentages is an average of triplicate preparation, based on the concentration of HRP at 404 and ϵ = 102,000 $M^{-1}cm^{-1}$.

(b) The number in brackets is the RZ value, the ratio of absorbance at 404 nm to absorbance at 278 nm; RZ value for the native enzyme is 3.2.

The decreased yield after dihydrazide reaction, could be explained mainly due to the Amicon membrane, where after filtering the dihydrazide treated enzyme, a yellowish (FAD) or brownish (hemin) layer of GO and HRP, respectively, were fixed on the membrane, some of which could be reclaimed by leaving buffer stirring in the Amicon cell overnight at 4°C. Yields could probably be improved by the use of a gel filtration column, but this would require more effort and would furnish diluted samples.

E. Preparation of the Enzyme Conjugates "GO-DH-HRP"

After overnight incubation of the GO-DH derivative and oxidized peroxidase at 4°C, the GO-Dihydrazone-HRP conjugate formed was cleaned up by ultrafiltration using 100,000 and 300,000 molecular weight exclusion membranes. The retained fraction was kept for characterization. This procedure permitted the removal of non-covalently bound peroxidase and glucose oxidase. The purified conjugate was then subjected to multicomponent analysis and activity tests.

1. Multicomponent Analysis

Multicomponent analysis is a program available as a software package on the Hewlett-Packard spectrophotometer. Analysis is based on the spectral differences of the two enzymes. Here, oxidized HRP and GO-DH were used as standards. Figure 10 shows the spectra of GO-DH, oxidized-HRP and the conjugate of both, before and after cleaning. The spectra of GO-DH and oxidized HRP adds up to give the spectrum of the conjugate, indicating that the spectral extinction coefficients do not change before and after conjugation, therefore, the results of multicomponent analysis may be reliable.

FIGURE 10

SPECTROPHOTOMETRIC SCANS OF CONJUGATED HRP AND GO

Legend

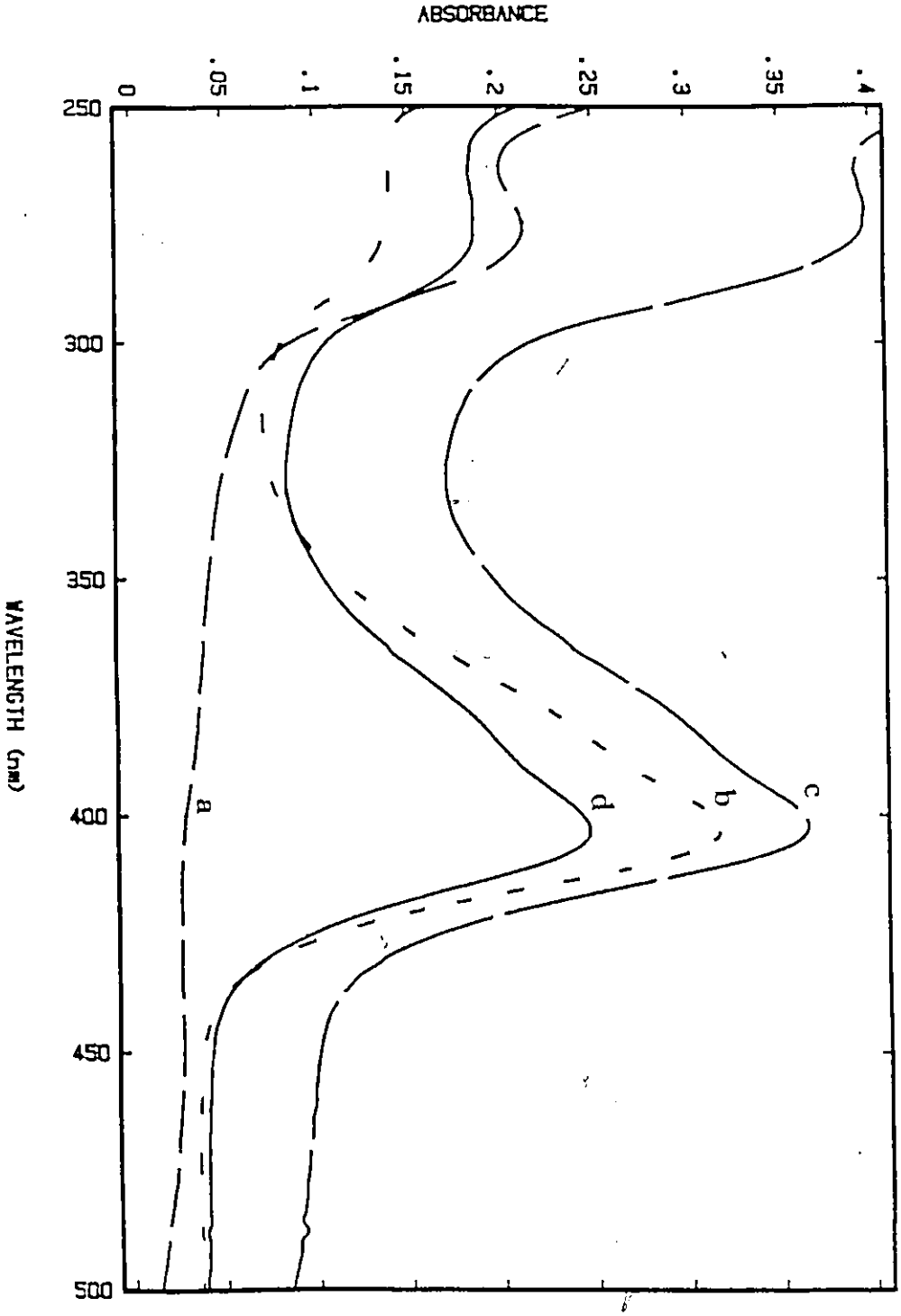
a. GO-DH used as a standard in multicomponent analysis. Concentration of GO was determined using λ_{450} nm and ϵ = 28,200 $M^{-1}cm^{-1}$.

b. Oxidized HRP was used as a standard for multicomponent analysis. Peroxidase concentration was determined by using λ = 404 nm, ϵ = 102,000 $M^{-1}cm^{-1}$.

c. The mixture of the same concentrations of GO-DH (a) and oxidized HRP (b) was incubated overnight at 4°C. This spectrum was taken before cleaning the mixture by ultrafiltration.

d. Conjugated enzymes GO-DH-HRP. This spectrum was taken after filtering the mixture by using Amicon membranes YM 100,000 and XM 300,000. The difference between c and d spectra is due to the loss of unconjugated GO-DH and oxidized HRP by ultrafiltration.

FIGURE 10


ABSORBANCE
0
.05
.1
.15
.2
.25
.3
.35
.4
250
300
350
400
450
500
WAVELENGTH (nm)
a
b
c
d

The absorbance of GO at 450 nm is so small as to be negligible at the concentrations used in conjugation experiments and, hence, is not as useful for quantitative analysis as might have been expected. To overcome such a conflict the protein peak (λ_{278}) which is quite large for both GO and HRP had to be included in the spectrum. The spectrum from 250 to 500 nm of a known concentration of each modified enzyme was stored and used as a standard for multicomponent analysis.

To show that the protein peak is relevant for accurate determination of the conjugated enzymes, known mixtures of unmodified HRP and GO were subjected to multicomponent analysis, using standards with a spectral range of 250 to 500 nm (Table VI). A mean recovery of 106.5 ± 4.7% and 101 ± 6.4% were obtained for both HRP and GO, respectively. Consequently, the estimated ratio was 105 ± 4.74% of the actual one (Table VI). The small increase in the percent recovery of both enzymes, would result in proportional underestimates of their specific activities.

For comparison, standards with a spectral range between 300 and 500 nm were used (Table VII). An undesirable high mean recovery of 121 ± 39% were obtained for GO, a reasonable mean recovery of 104 ± 2.33% for HRP,with the consequence that the estimated ratio was 91.5 ± 23.3% of the actual one.

2. Activity Tests

The efficiency of the conjugation procedure is presented in Table VIII. The amount of peroxidase conjugated to GO-DH (Table VIII) corresponded to the number of hydrazides incorporated into glucose oxidase (Table II). However, it seems a maximum of 19 moles of oxidized HRP can be conjugated to one

TABLE VI

EVALUATION STUDY OF MULTICOMPONENT ANALYSIS EMPLOYING STANDARDS[a] OF HRP AND GO WITH SPECTRA BETWEEN 250–500 nm

Actual[b] ratio of HRP/GO	Ratio estimated by multicomponent analysis	% recovery[c] of HRP	% recovery of GO
3.157	3.313 ± 0.002	105.47 ± 0.02	100.75 ± 0.04
3.955	4.050 ± 0.002	113.41 ± 0.02	110.6 ± 0.04
6.310	6.590 ± 0.005	108.33 ± 0.02	103.11 ± 0.05
9.022	9.520 ± 0.008	103.17 ± 0.02	97.83 ± 0.08
15.789	17.121 ± 0.021	102.01 ± 0.02	93.20 ± 0.11

(a) Concentrations of HRP and GO standards were 8.74 and 3.83 μM, respectively.

(b) Concentrations of HRP and GO before mixing were determined by spectrophotometric analysis λ_{404}, ϵ = 102,000 $M^{-1}cm^{-1}$ and λ_{450}, ϵ = 28,200 $M^{-1}cm^{-1}$, respectively.

(c) Concentrations of enzymes were obtained by multicomponent analysis and reported as percentages of the calculated ones.

TABLE VII

EVALUATION OF MULTICOMPONENT ANALYSIS EMPLOYING STANDARDS[a] OF HRP AND GO WITH SPECTRA BETWEEN 300-500 nm

Actual[b] Ratio HRP/GO	Ratio estimated by multicomponent analysis	% recovery[c] of HRP	% recovery of GO
3.157	-	-	-
3.955	2.340 ± 0.003	105.65 ± 0.03	177.9 ± 0.10
6.310	5.737 ± 0.001	106.51 ± 0.02	117.2 ± 0.14
9.022	10.250 ± 0.026	102.50 ± 0.02	90.25 ± 0.23
15.789	16.104 ± 0.059	102.05 ± 0.02	100.04 ± 0.30

(a) Concentrations of HRP and GO standards were 8.74 and 3.83 μM, respectively.

(b) Concentrations of HRP and GO before mixing were determined spectrophotometrically with λ_{404}, ϵ = 102,000 $M^{-1}cm^{-1}$ and λ_{430}, ϵ = 28,200 $M^{-1}cm^{-1}$, respectively.

(c) Concentrations of enzymes were obtained by multicomponent analysis and reported as percentages of the actual ones.

TABLE VIII

CHARACTERIZATION OF GLUCOSE OXIDASE-DIHYDRAZONE-PEROXIDASE CONJUGATES

Prep. no.	$NaIO_4$ mM	HRP/GO[a]	HRP/GO[b]	k_{cat}[c] for GO min^{-1}	k_{cat}[c] for HRP min^{-1}	k_{cat}[c] for the conjugate min^{-1}
1	4	8.6	11.7±0.5	13013±361 (102)[d][156][e]	3778±90 (64)[70]	12777±348
2	4	10.7	16.7±0.7	9025±139 (66) [82]	3855±70 (65) [85]	8900±110
3	6	8.3	18.8±0.6	11377±250 (81) [102]	3486±30 (74) [88]	11103±350
4	10	7.3	13.0±1.5	8926±158 (74)[109]	3977±80 (69) [76]	8838±60

(a) Ratio at zero time incubation of the concentrations of oxidized HRP to GO-DH.

(b) Ratio of the concentrations of oxidized-HRP to GO-DH after 18-h incubation and filtering by ultrafiltration, using 100,000 and 300,000 molecular weight exclusion membranes. Concentrations were calculated using the multicomponent analysis, available as software with the Hewlett Packard spectrophotometer. Analysis is based on the spectral differences of the two enzymes. Standards used were GO-DH and oxidized-HRP, their spectra from 250-500 nm with their respective concentrations were stored in memory.

(c) k_{cat}, the catalytic constant or turnover number was calculated using the ratio of the maximum velocity to that of the enzyme concentration. Enzyme activity was calculated by the oxidative coupling of HDCBS and 4-AAP. GO activity was determined in the presence of free peroxidase and 100 mM glucose; HRP assays were completed in the presence of 0.1 mM H_2O_2. The activity of the conjugate represents the activity of GO-HRP conjugate, using glucose as a substrate, and no free peroxidase was added. All assays were performed in triplicate.

TABLE VII cont'd

(d) Numbers in round brackets, are the relative specific activities of the enzymes as a percentage of the k_{cat} of the native enzyme for the respective preparations given in Table II.

(e) Numbers in square brackets, are the relative specific activities of the conjugated GO and HRP, as a percentage of the k_{cat} of their previous modified form for the respective preparations given in Table II and III, respectively.

mole of GO-DH, even with the incorporation of 31 moles of adipic dihydrazide to one GO (preparation number 3 Tables II and VIII). The yield of conjugated enzymes after ultrafiltration were averages of 50 and 30% for HRP and GO, respectively, compared to their respective concentrations before ultrafiltration. That explains why the molar ratios of HRP to GO after conjugation and ultrafiltration were greater than the calculated ones at zero time incubation (Table VIII).

For one of the preparations (number 3), the activity tests were performed on a GO-DH and oxidized HRP mixture at zero time and after overnight incubation but before the conjugation mixture was filtered by the Amicon membrane. The activity of HRP decreased by 12 ± 1%, while glucose oxidase activity was practically unchanged (1.2% more active). This relative specific activity change of conjugated HRP was similar to the change on going from oxidized HRP to HRP-DH (Table VIII square brackets, and III last column). The opposite was obtained for the relative specific activities of the conjugated (GO Table VIII), compared to their respective activities in the previous modified form, GO-DH (Table II), especially for preparations one and two where the specific activity increased and decreased respectively and stayed almost the same for the last two preparations (Table VIII). From these results it was desirable that a method independent of the multicomponent analysis might be developed for estimating HRP/GO ratios based on activity measurements. This method would be based on assumptions regarding changes in specific activities of the two modified enzymes being conjugated. For example, if the specific activity of conjugated GO was the same as its previously modified form (GO-DH; Table II), and if the activity of conjugated HRP did not change from the HRP-DH form (Table III), the molar ratios of HRP to GO for preparations one to four would be 7.0±0.3, 20.5±0.9, 19.2±0.9, 13.5±0.3, respectively.

Comparing these results with the ones obtained from multicomponent analysis (Table VIII), it is noticed that the results of multicomponent analysis overestimated the ratio in preparation 1, mainly because it underestimated the conjugated GO concentration by 50%. The ratio was underestimated for preparation 2, mainly because of overestimation of the GO concentration by multicomponent analysis by 20%. However, in this preparation there were only 17 of ADH conjugated to one GO (Table II), making a ratio of 20.5±0.9 of HRP to GO impossible. Therefore, based on the above assumptions, preparation two would not fit the data. For the last two preparations, the ratios obtained from the multicomponent analysis and activity tests were in the same range. The main conclusion from consideration of both of these means of estimating HRP/GO ratios in the conjugates is that neither gives an unambiguous estimate of the ratio but both are indicative of approximate ratios.

The percent decrease of relative specific activities of oxidized peroxidase upon conjugation to GO-DH (Table VIII) were the same as that for HRP-DH (Table III). That might mean the results of multicomponent analysis concerning HRP is more reliable than glucose oxidase results for reasons discussed previously. However, a range of 74% to 64% of the relative specific activity was maintained for oxidized HRP conjugated GO-DH.

The activities of glucose oxidase conjugated to peroxidase (k_{cat} for GO) (Table VIII) where the activity test were done by the addition of free HRP are the same as those of the conjugates (k_{cat} of the conjugates) where no free peroxidase was added. Since all the preparations of Table VIII have a high conjugated HRP ratio compared to GO, two other preparations were performed having lower ratios of HRP conjugated to GO (Table IX). Therefore, when the conjugated peroxidase and glucose oxidase are in a ratio of two or more (Table IX) the activities of GO conjugated and the conjugate were the same. From the combined results of Tables VIII and IX it may be concluded that when HRP conjugated to GO-DH in a ratio of two and over no free peroxidase is needed for assaying the activity of the conjugate.

TABLE IX

ACTIVITY ON CONJUGATED GLUCOSE OXIDASE AND THE CONJUGATE (GO-DH-HRP)

[HRP]/[GO][a]	Activity[b] of conjugated GO U/L	Total activity[b] of the conjugate U/L
2.217±0.002	9.58 ± 0.02	9.50 ± 0.04
3.578±0.002	17.95 ± 0.03	17.70 ± 0.01

a) The concentration of conjugated HRP and GO were determined by multicomponent analysis, after GO-DH and oxidized peroxidase been incubated overnight at 4°C and then cleaned up by ultrafiltration using 100,000 and 300,000 membranes.

(b) Enzyme activity in U/L was calculated in triplicate by oxidative coupling of HDCBS and 4-AAP. Activity of conjugated GO was determined in the presence of excess peroxidase and 100 mM glucose. Activity of the conjugate (GO-DH-HRP) was performed with glucose as a substrate but without free peroxidase.

For comparison, a control was run using different ratios of native peroxidase and glucose oxidase (Table X). In this case when the ratio of the concentration of peroxidase to GO was less than four, extra peroxidase must be added for the activity assay of the enzymes mixture. At a ratio of four, the activity test of the mixture shows a lag phase of one and a half minutes after which the activity reaches that of glucose oxidase alone (with free HRP).

F. Kinetic Study

The results of steady-state kinetic investigations for native and conjugated glucose oxidase are shown in Figure 11. Literature values for K_m of 33 mM have been reported using an oxygen electrode for detection of native enzyme (36,61). Nakamura et al. (32) have reported a K_m of 28 mM for the native enzyme. Our study demonstrated a lower K_m value for native glucose oxidase of 17.25 ± 1.43 mM utilizing glucose as a substrate. A previous study performed in our laboratory using the same chromogen system, reported a K_m of 13.3±2.55 mM (25). All glucose oxidase K_m's calculated in this and previous (25) work from this laboratory are based on total glucose concentration. The dissimilarities in the methods of quantitation may account for the difference from the literature value. The K_m of glucose oxidase conjugated to peroxidase, where 6 mM periodate had been used, was 16.2 ± 0.9 mM, with the addition of free HRP to assay mixture and was 16.1 ± 0.5 mM, when no free peroxidase was added (Figure 11).

The Michaelis constant derived in the literature for the native HRP was 40 μM for H_2O_2 utilizing o-dianisidine as chromogen (52). This work demonstrated a K_m of 25.07 ± 4.07 μM for H_2O_2 with native HRP while conjugated peroxidase showed a K_m as low as 14.35 ± 1.70 μM (Figure 12). Again previous work in our laboratory (25), indicated a K_m of 59±5 μM for native HRP using the same chromogen system.

For the different preparations of enzyme conjugates the kinetic parameters were determined by non-linear regression and are shown in Tables XI and XII

TABLE X

ACTIVITY ON MIXTURES OF NATIVE HRP AND GO IN DIFFERENT RATIOS

Ratio of [HRP]/[GO][a]	Activity of GO U/L[b]	Activity for the mixture U/L[c]
1.14	18.97 ± 0.03	9.60 ± 0.50
2.28	19.36 ± 0.3	15.55 ± 0.05
3.17	13.33 ± 0.08	11.99 ± 0.14
3.94	7.20 ± 0.09	6.34 ± 0.08
6.33	14.53 ± 0.02	14.58 ± 0.03
9.05	14.50 ± 0.07	14.31 ± 0.02
15.84	14.63 ± 0.08	14.62 ± 0.09

(a) Concentrations of GO and HRP were determined spectrophotometrically.

(b) Enzyme activity in units was calculated in triplicate using a solution containing 9 mM HDCBS, 2.4 mM 4-AAP, 100 mM glucose and 3 units of extra HRP per mL.

(c) Activity of the mixture was determined as above by using glucose as a substrate but no extra HRP was added.

FIGURE 11

LINEWEAVER-BURK PLOTS OF KINETIC DATA FOR NATIVE AND CONJUGATED GLUCOSE OXIDASE

Legend

Double reciprocal plots of data collected for the native (rectangles) and conjugated (solid triangles) glucose oxidase (preparation no. 3) are shown. Initial rates were collected by colorimetric assay for GO activity using 9.0 mM HDCBS, 2.4 mM 4-AAP and 2 units of extra HRP in a 1.0 mL volume. Substrate glucose concentrations varied from 2 to 70 mM. Similarly, an analysis of the initial rates of the conjugate (GO-DH-HRP) with no addition of extra peroxidase was carried out (open triangles). All assays were in duplicate and calculation of the parameters K_m and V_{max} was carried out by non-linear regression analysis of the initial velocity vs substrate concentration data. Double reciprocal plots were chosen simply for graphic presentation.

K_m for native GO was calculated to be 17.3 ± 1.4 mM and V_{max} = 12.68 ± 0.43 μM min^{-1} (r = 0.9988).

The Michaelis constant obtained for conjugated GO where free HRP was added were: K_m = 16.2 ± 0.9 mM, V_{max} = 6.44 ± 0.13 μM min^{-1} and a correlation coefficient of 0.9988.

The conjugated system where no free HRP was added gave the following kinetic data: K_m = 16.1 ± 0.5 mM, V_{max} = 6.37 ± 0.06 μM min^{-1} and r = 0.9997.

FIGURE 11

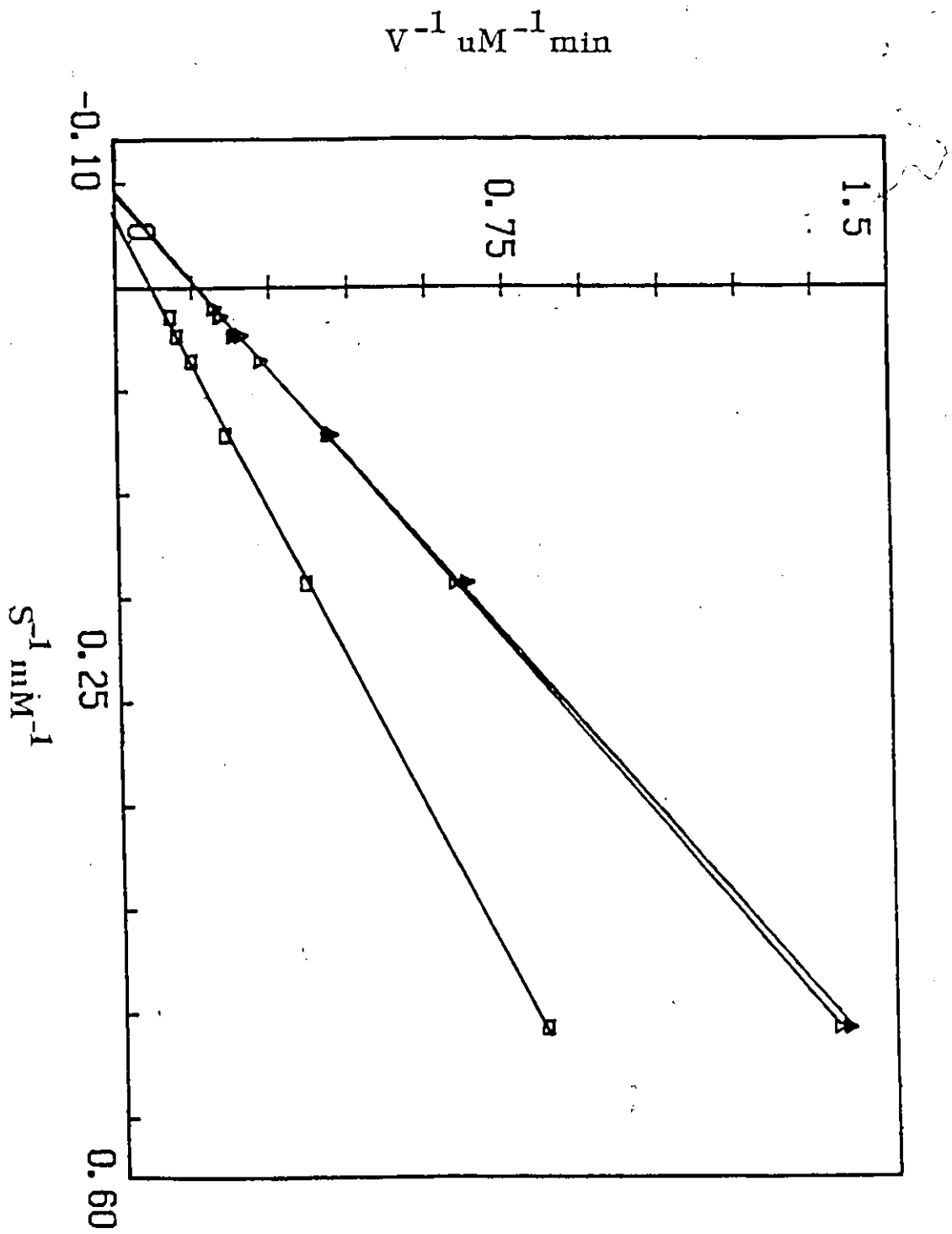


FIGURE 12

LINEWEAVER-BURK PLOTS OF KINETIC DATA FOR NATIVE AND CONJUGATED PEROXIDASE

Legend

Double reciprocal plots of data collected for the native (rectangles) and conjugated (triangles) peroxidase (preparation no. 3) are shown.

Duplicate assays were completed as described in the Experimental section for peroxidase activity but using peroxide concentrations between 2 and 50 μM.

The kinetic data presented here was calculated by non-linear regression. For native enzyme Km = 25.07 ± 4.07 μM, Vmax = 10.06 ± 0.66 μM min^{-1} and a correlation coefficient (r = 0.991).

Kinetic constants obtained for conjugated peroxidase were: Km = 14.34 ± 1.7 μM, V_m = 6.80 ± 0.29 μM min^{-1} and r = 0.996.

FIGURE 12

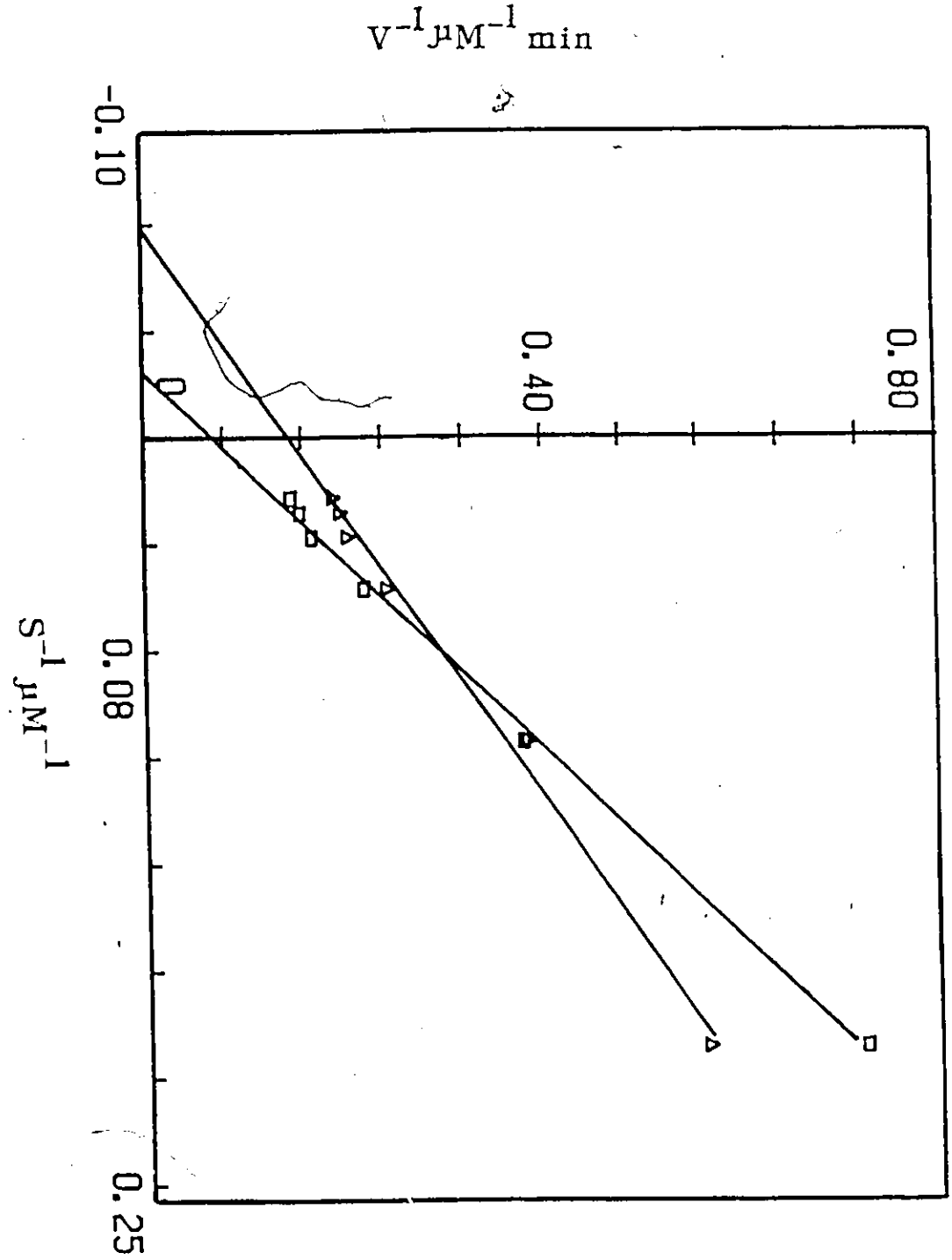


TABLE XI

KINETIC DATA FOR CONJUGATED[a] GLUCOSE OXIDASE AND THE CONJUGATED SYSTEM

Prep. No.	Activity determined	$NaIO_4$ (mM)	K_m (mM±SD)	k_{cat}[b] min^{-1} (min^{-1})
	Native	-	17.3 ± 1.4	11963±411
1	GO	4	16.4 ± 0.8	15348±605 (128)[c]
	Conjugate (d)	4	13.6 ± 1.2	14182±726
2	GO	4	14.5 ± 1.1	8385±243 (70)
	Conjugate	4	18.4 ± 1.7	9675±264
3	GO	6	16.2 ± 0.9	11673±287 (98)
	Conjugate	6	16.14 ± 0.5	11547±162
4	GO	10	17.1 ± 0.9	8800±209 (74)
	Conjugate	10	16.2 ± 0.3	8154±99

(a) kinetic data was determined by nonlinear regression.

(b) k_{cat} was calculated using the ratio of Vmax (μmoles $min^{-1}mL^{-1}$) to enzyme concentration (μmoles mL^{-1}). Enzyme concentrations determined by multicomponent analysis were in the range 0.5 to 1.3 nM. Assays were run in duplicate.

(c) numbers in brackets are the relative specific activity of the enzyme as a percentage of the k_{cat} of the native.

(d) Activity on the conjugate was made without the addition of free HRP.

TABLE XII

KINETIC DATA FOR CONJUGATED PEROXIDASE[a]

Preparation	$NaIO_4$ (mM)	K_m (μM ± SD)	k_{cat}[b] (min^{-1})
Native HRP	-	25.1 ± 4.1	7292±481
Conjugate 1	4	20.10 ± 3.4	4810±397 (66)[c]
Conjugate 2	4	19.7 ± 4.5	5600±493 (77)
Conjugate 3	6	14.3 ± 1.7	5113±225 (70)
Conjugate 4	10	25.7 ± 2.0	5600±387 (77)

(a) Kinetic data was determined by non-linear regression.

(b) k_{cat} was calculated using the ratio of V_{max} (μmoles $min^{-1}mL^{-1}$) to enzyme concentration (μmoles mL^{-1}). Enzyme concentrations determined by multicomponent analysis were in the range 0.9 to 2.0 mM. Assays were run in duplicate.

(c) Numbers in brackets, are the relative specific activity of the enzyme as a percentage of the k_{cat} of the native.

For the different preparations of enzyme conjugates the kinetic parameters were determined by non-linear regression and are shown in Tables XI and XII for glucose oxidase and peroxidase, respectively. Low K_m values for conjugated HRP were obtained through preparations 1 to 3 (Table XII). This could be due to the enhancement and optimization of the intramolecular distance and orientation of the enzyme upon conjugation, which increase the accessibility of H_2O_2 formed by GO to the active site of HRP. However, the K_m for conjugated glucose oxidase for all preparation was constant. Overall, the relative specific activities of both conjugated enzymes compared to the respective native forms were maintained at an average of 92 and 72% for GO and HRP respectively (Tables XI and XII).

G. Stability of the Conjugates

Kinetic analysis of the conjugated enzymes were repeated after 2 months. Both peroxidase and glucose oxidase showed no alteration in their a catalytic properties. V_{max} and K_m gave same results as that of the day of preparation. Therefore, we conclude that the conjugated enzymes were stable in 0.1 M phosphate buffer pH 7.5 at $4^{o}C$ for at least 2 months.

CHAPTER IV

SUMMARY AND CONCLUSION

Our study was primarily concerned with the development of a method for the conjugation of HRP and GO, through their carbohydrate moieties. Mild periodate oxidation resulted in an average of 85% recovery of the relative specific activity for both enzymes.

Conjugation of periodate-treated HRP and GO was effected through hydrazone formation with adipic dihydrazide, which is stable at neutral pH and does not require reduction with sodium cyanoborohydride.

Under the conditions described in the Experimental section, the conjugated enzymes maintained an average relative specific activity of 72% and 92% for peroxidase and glucose oxidase, respectively.

The K_m for conjugated glucose oxidase was the same as the native enzyme and constant through all the preparations. However, the K_m for conjugated peroxidase was lower than the native, where the lowest K_m obtained by using 6 mM periodate for enzymes oxidation. Enzymes conjugates proved to be stable for at least 2 months at $4^{o}C$.

It should be noted that the method of preparing enzyme conjugates described here, can be used for labelling an immunoglobulin with a reporter molecule such as peroxidase. An IgG-DH-HRP conjugate utilizing the method described herein, is already in progress in our laboratory.

APPENDIX

DATA FROM KINETIC STUDIES

Assays were done in duplicate.

A. Peroxidase

Native Peroxidase

Substrate μM	Rate μM min^{-1}
5	1.38/1.40
10	2.62/2.65
20	4.50/4.55
30	5.88/5.90
50	6.73/6.71
70	7.10/7.12

Conjugated Peroxidase: Preparation 1

Substrate μM	Rate μM min^{-1}
5	0.84/0.86
10	1.40/1.42
20	2.41/2.44
30	2.79/2.71
50	3.26/3.11

Prep. 1. Conjugated peroxidase: same preparation as above, kinetic study were repeated 2 months later.

Substrate μM	Rate μM min^{-1}
5	0.87/0.89
10	1.41/1.42
20	2.45/2.48
30	2.83/2.87
50	3.26/3.26
70	3.33/3.32

Prep. 2

Substrate μM	Rate μM min^{-1}
5	1.43/1.44
10	2.97/2.98
20	5.05/5.08
30	6.22/6.5
50	6.80/6.82
70	7.05/7.15

Prep. 3

Substrate μM	Rate μM min^{-1}
2	0.86/0.86
5	1.74/1.78
10	2.7/2.6
20	4.24/3.86
30	4.80/4.86
50	5.17/5.05

Prep. 4

Substrate μM	Rate μM min^{-1}
2	0.71/0.73
5	1.71/1.70
10	3.5/3.45
20	5.32/5.24
30	5.82/6.03
40	6.67/6.63
50	7.31/7.3

Correlation coefficients for all kinetic data were between 0.991-0.996.

Glucose Oxidase

Native GO (with extra HRP)

Substrate mM	Rate μM min^{-1}
2	1.22/1.21
5	2.77/2.76
10	4.77/4.70
20	6.74/6.77
30	8.33/8.40
50	9.26/9.24
70	10.10/10.23

Preparation 1: Conjugated Glucose Oxidase (with free HRP)

Substrate mM	Rate $\mu M\ min^{-1}$
2	1.43/1.46
5	3.33/3.16
10	5.18/5.35
20	7.51/7.49
30	8.80/8.81
50	10.60/10.70
70	11.03/11.10

Preparation 1: Conjugate (without free HRP)

Substrate mM	Rate $\mu M\ min^{-1}$
2	1.40/1.48
5	3.27/3.28
10	5.25/5.30
20	7.81/7.83
30	9.10/9.11
50	10.09/10.07
70	10.38/10.39

Preparation 2: Conjugated GO (with free HRP)

Substrate mM	Rate $\mu M\ min^{-1}$
2	0.70/0.72
5	1.48/1.46
10	2.39/2.42
20	3.53/3.55
30	4.33/4.33
50	4.89/4.81
70	5.14/5.09

Conjugate: (without free HRP)

Substrate mM	Rate $\mu M\ min^{-1}$
2	0.71/0.73
5	1.53/1.53
10	2.44/2.45
20	3.55/3.56
30	4.07/4.09
50	4.82/4.85
70	5.09/5.92

Preparation 3: Conjugated GO (with free HRP)

Substrate mM	Rate $\mu M\ min^{-1}$
2	1.10/1.18
5	2.40/2.44
10	4.19/4.21
20	6.34/6.35

30	7.28/7.29
50	8.53/8.56

Conjugate: (without free HRP)

Substrate mM	Rate $\mu M\ min^{-1}$
2	1.16/1.13
5	2.44/2.48
10	4.13/4.07
20	5.94/5.92
30	6.80/6.86
50	8.01/8.05
70	8.60/8.63

Preparation 4: Conjugated GO (with free HRP)

Substrate mM	Rate $\mu M\ min^{-1}$
2	0.72/0.72
5	1.57/1.58
10	2.69/2.72
20	3.98/3.98
30	4.55/4.54
50	5.17/5.19
70	5.39/5.36

Conjugate: (without free HRP)

Substrate mM	Rate $\mu M\ min^{-1}$
2	0.70/0.72
5	1.53/1.47

10	2.84/2.80
20	4.01/4.02
30	4.89/4.91
50	5.70/5.6
70	6.05/6.09

Correlation coefficient for all kinetic data presented above for conjugated GO and the conjugated system were between 0.9981-0.9999.

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